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| (54) Title: NOVEL OSTEOINDUCTIVE COMPOSITIONS (57) Abstract Human and bovine bone inductive factor products and processes. The factors may be produced by recombinant techniques and are useful in the research and treatment of bone and periodontal defects. | | |

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NOVEL OSTEOINDUCTIVE COMPOSITIONS

The present invention relates to novel proteins and processes for obtaining them. These proteins are capable of inducing cartilage and bone formation.

Background

Bone is a highly specialized tissue characterized by an extensive matrix structure formed of fibrous bundles of the protein collagen, and proteoglycans, noncollagenous proteins, lipids and acidic proteins. The processes of bone formation and renewal/repair of bone tissue, which occur continuously throughout life, are performed by specialized cells. Normal embryonic long bone development is preceded by formation of a cartilage model. Bone growth is presumably mediated by "osteoblasts" (bone-forming cells), while remodeling of bone is apparently accomplished by the joint activities of bone-resorbing cells, called "osteoclasts" and osteoblasts. A variety of osteogenic, cartilage-inducing and bone inducing factors have been described. See, e.g. European patent applications 148,155 and 169,016 for discussions thereof.

Brief Description of the Invention

The present invention provides novel proteins in purified form. Specifically, four of the novel proteins are designated BMP-1, BMP-2 Class I (or BMP-2), BMP-3, and BMP-2 Class II (or BMP-4) wherein BMP is bone morphogenic protein. These proteins are characterized by peptide sequences the same as or substantially homologous to amino acid sequences illustrated in Tables II through VIII below. They are capable of inducing bone formation at a predetermined site. These bone inductive factors are further characterized by biochemical and biological characteristics including activity at a concentration of 10 to 1000ng/gram of bone in an in vivo rat bone formation assay described below. Proteins of this invention may be encoded by the DNA sequences depicted in the Tables or by sequences capable

of hybridizing thereto and coding for polypeptides with bone growth factor biological properties or other variously modified sequences demonstrating such properties.

One of the proteins of the invention is designated BMP-1. A portion of the human BMP-1 or hBMP-1 is characterized by the same or substantially the same peptide sequence as that of amino acid #1 through amino acid #37 of Table V, below which represents a genomic hBMP-1 fragment or amino acid #1 through amino acid #730 of Table VI which represents the hBMP-1 cDNA. hBMP-1 or a related bone inductive factor may be further characterized by at least a portion of these sequences. These peptide sequences are encoded by the same or substantially the same DNA sequence, as depicted in nucleotide #3440 through nucleotide #3550 of Table V and in nucleotide #36 through nucleotide #2225 of Table VI, respectively. These hBMP-1 polypeptides are further characterized by the ability to induce bone formation. hBMP-1 demonstrates activity in an in vivo rat bone formation assay at a concentration of 10 to 1000ng/gram of bone.

The homologous bovine growth factor of the invention, designated bBMP-1, is characterized by a peptide sequence containing the same or substantially the same sequence as that of amino acid #1 through amino acid #37 of Table II below which represents a genomic bBMP-1 fragment. This peptide sequence is encoded by the same or substantially the same DNA sequence as depicted in nucleotide #294 through nucleotide #404 of Table II. The bovine peptide sequence identified in Table II below is also 37 amino acids in length. bBMP-1 is further characterized by the ability to induce bone formation.

Another bone inductive protein composition of the invention is designated BMP-2 Class I (or BMP-2). It is characterized by at least a portion of a peptide sequence the same or substantially the same as that of amino acid #1 through amino acid #396 of Table VII which represents the cDNA hBMP-2 Class I. This peptide sequence is encoded by the same or

substantially the same DNA sequence, as depicted in nucleotide #356 through nucleotide #1543 of Table VII. The human peptide sequence identified in Table VII is 396 amino acids in length. hBMP-2 or related bone inductive proteins may also be characterized by at least a portion of this peptide sequence. hBMP-2 Class I is further characterized by the ability to induce bone formation.

The homologous bovine bone inductive protein of the invention designated bBMP-2 Class I (or bBMP-2), has a DNA sequence identified in Table III below which represents the genomic sequence. This bovine DNA sequence has a prospective 129 amino acid coding sequence followed by approximately 205 nucleotides (a presumptive 3' non-coding sequence). bBMP-2, Class I is further characterized by the ability to induce bone formation. A further bone inductive protein composition of the invention is designated BMP-2 Class II or BMP-4. The human protein hBMP-2 Class II (or hBMP-4) is characterized by at least a portion of the same or substantially the same peptide sequence between amino acid #1 through amino acid #408 of Table VIII, which represents the cDNA of hBMP-2 Class II. This peptide sequence is encoded by at least a portion of the same or substantially the same DNA sequence as depicted in nucleotide #403 through nucleotide #1626 of Table VIII. This factor is further characterized by the ability to induce bone formation.

Still another bone inductive factor of the invention, BMP-3, is represented by the bovine homolog bBMP-3. bBMP-3 is characterized by the DNA sequence and amino acid sequence of Table IV A and B which represents the bovine genomic sequence. It is characterized by at least a portion of a peptide sequence the same or substantially the same as amino acid #1 through amino acid #175 of Table IV A and B. BMP-3 is further characterized by the ability to induce bone formation. The bovine factor may be employed as a tool for obtaining the analogous human BMP-3 protein or other mammalian bone inductive proteins. The proper characterization of this bovine bone

inductive factor provides the essential "starting point" for the method employing this sequence. The method, employing techniques known to those skilled in the art of genetic engineering, involves using the bovine DNA sequence as a probe to screen a human genomic or cDNA library; and identifying the DNA sequences which hybridize to the probes. A clone with a hybridizable sequence is plaque purified and the DNA isolated therefrom, subcloned and subjected to DNA sequence analysis. Thus as another aspect of this invention is a human protein hBMP-3, produced by this method.

Another aspect of the invention provides pharmaceutical compositions containing a therapeutically effective amount of one or more bone growth factor polypeptides according to the invention in a pharmaceutically acceptable vehicle. These compositions may further include other therapeutically useful agents. They may also include an appropriate matrix for delivering the proteins to the site of the bone defect and for providing a structure for bone growth. These compositions may be employed in methods for treating a number of bone defects and periodontal disease. These methods, according to the invention, entail administering to a patient needing such bone formation an effective amount of at least one of the novel proteins BMP-1, BMP-2 Class I, BMP-2 Class-II, and BMP-3 as described herein.

Still a further aspect of the invention are DNA sequences coding on expression for a human or bovine polypeptide having the ability to induce bone formation. Such sequences include the sequence of nucleotides in a 5' to 3' direction illustrated in Tables II through VIII. Alternatively, a DNA sequence which hybridizes under stringent conditions with the DNA sequences of Tables II - VIII or a DNA sequence which hybridizes under non-stringent conditions with the illustrated DNA sequences and which codes on expression for a protein having at least one bone growth factor biological property are included in the present invention. Finally, allelic or other variations of the

sequences of Tables II through VIII, whether such nucleotide changes result in changes in the peptide sequence or not, are also included in the present invention.

Still a further aspect of the invention is a vector containing a DNA sequence as described above in operative association with an expression control sequence. Such vector may be employed in a novel process for producing a bone growth factor polypeptide in which a cell line transformed with a DNA sequence encoding expression of a bone growth factor polypeptide in operative association with an expression control sequence therefor, is cultured. This claimed process may employ a number of known cells as host cells for expression of the polypeptide. Presently preferred cell lines are mammalian cell lines and bacterial cells.

Other aspects and advantages of the present invention will be apparent upon consideration of the following detailed description and preferred embodiments thereof.

Detailed Description of the Invention

The proteins of the present invention are characterized by amino acid sequences or portions thereof the same as or substantially homologous to the sequences shown in Tables II - VIII below. These proteins are also characterized by the ability to induce bone formation.

The bone growth factors provided herein also include factors encoded by the sequences similar to those of Tables II - VIII, but into which modifications are naturally provided (e.g. allelic variations in the nucleotide sequence which may result in amino acid changes in the polypeptide) or deliberately engineered. For example, synthetic polypeptides may wholly or partially duplicate continuous sequences of the amino acid residues of Tables II - VIII. These sequences, by virtue of sharing primary, secondary, or tertiary structural and conformational characteristics with bone growth factor polypeptides of Tables II - VIII may possess bone growth factor biological properties in common therewith. Thus, they may be

employed as biologically active substitutes for naturally-occurring bone growth factor polypeptides in therapeutic processes.

Other specific mutations of the sequences of the bone growth factors described herein involve modifications of one or both of the glycosylation sites. The absence of glycosylation or only partial glycosylation results from amino acid substitution or deletion at one or both of the asparagine-linked glycosylation recognition sites present in the sequences of the bone growth factors shown in Tables II-VIII. The asparagine-linked glycosylation recognition sites comprise tripeptide sequences which are specifically recognized by appropriate cellular glycosylation enzymes. These tripeptide sequences are either asparagine-X-threonine or asparagine-X-serine, where X is usually any amino acid. A variety of amino acid substitutions or deletions at one or both of the first or third amino acid positions of a glycosylation recognition site (and/or amino acid deletion at the second position) results in non-glycosylation at the modified tripeptide sequence.

The present invention also encompasses the novel DNA sequences, free of association with DNA sequences encoding other proteinaceous materials, and coding on expression for bone growth factors. These DNA sequences include those depicted in Tables II - VIII in a 5' to 3' direction and those sequences which hybridize under stringent hybridization conditions [see, T. Maniatis et al, Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory (1982), pages 387 to 389] to the DNA sequences of Tables II - VIII.

DNA sequences which hybridize to the sequences of Tables II - VIII under relaxed hybridization conditions and which code on expression for bone growth factors having bone growth factor biological properties also encode bone growth factors of the invention. For example, a DNA sequence which shares regions of significant homology, e.g., sites of glycosylation

or disulfide linkages, with the sequences of Tables II - VIII and encodes a bone growth factor having one or more bone growth factor biological properties clearly encodes a member of this novel family of growth factors, even if such a DNA sequence would not stringently hybridize to the sequence of Tables II - VIII.

Similarly, DNA sequences which code for bone growth factor polypeptides coded for by the sequences of Tables II - VIII, but which differ in codon sequence due to the degeneracies of the genetic code or allelic variations (naturally-occurring base changes in the species population which may or may not result in an amino acid change) also encode the novel growth factors described herein. Variations in the DNA sequences of Tables II - VIII which are caused by point mutations or by induced modifications to enhance the activity, half-life or production of the polypeptides encoded thereby are also encompassed in the invention.

Another aspect of the present invention provides a novel method for producing the novel osteoinductive factors. The method of the present invention involves culturing a suitable cell or cell line, which has been transformed with a DNA sequence coding on expression for a novel bone growth factor polypeptide of the invention, under the control of known regulatory sequences. Suitable cells or cell lines may be mammalian cells, such as Chinese hamster ovary cells (CHO). The selection of suitable mammalian host cells and methods for transformation, culture, amplification, screening and product production and purification are known in the art. See, e.g., Gething and Sambrook, Nature, 293:620-625 (1981), or alternatively, Kaufman et al, Mol. Cell. Biol., 5(7):1750-1759 (1985) or Howley et al, U.S. Patent 4,419,446. Another suitable mammalian cell line, which is described in the accompanying examples, is the monkey COS-1 cell line. A similarly useful mammalian cell line is the CV-1 cell line.

Bacterial cells are suitable hosts. For example, the

various strains of E. coli (e.g., HB101, MC1061) are well-known as host cells in the field of biotechnology. Various strains of B. subtilis, Pseudomonas, other bacilli and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present invention. Additionally, where desired, insect cells may be utilized as host cells in the method of the present invention. See, e.g. Miller et al, Genetic Engineering, 8:277-298 (Plenum Press 1986) and references cited therein.

Another aspect of the present invention provides vectors for use in the method of expression of these novel osteoinductive polypeptides. Preferably the vectors contain the full novel DNA sequences described above which code for the novel factors of the invention. Additionally the vectors also contain appropriate expression control sequences permitting expression of the bone inductive protein sequences. Alternatively, vectors incorporating modified sequences as described above are also embodiments of the present invention and useful in the production of the bone inductive proteins. The vectors may be employed in the method of transforming cell lines and contain selected regulatory sequences in operative association with the DNA coding sequences of the invention which are capable of directing the replication and expression thereof in selected host cells. Useful regulatory sequences for such vectors are known to one of skill in the art and may be selected depending upon the selected host cells. Such selection is routine and does not form part of the present invention.

A protein of the present invention, which induces bone growth in circumstances where bone is not normally formed, has application in the healing of bone fractures. An osteogenic preparation employing one or more of the proteins of the invention may have prophylactic use in closed as well as open

fracture reduction and also in the improved fixation of artificial joints. De novo bone formation induced by an osteogenic agent contributes to the repair of congenital, trauma induced, or oncologic resection induced craniofacial defects, and also is useful in cosmetic plastic surgery. An osteogenic factor of the invention may be valuable in the treatment of periodontal disease, and in other tooth repair processes. Such agents may provide an environment to attract bone-forming cells, stimulate growth of bone-forming cells or induce differentiation of progenitors of bone-forming cells. Of course, the proteins of the invention may have other therapeutic uses.

A further aspect of the invention is a therapeutic method and composition for repairing fractures and other conditions related to bone defects or periodontal diseases. Such a composition comprises a therapeutically effective amount of at least one of the bone inductive factor proteins of the invention. The bone inductive factors according to the present invention may be present in a therapeutic composition in admixture with a pharmaceutically acceptable vehicle or matrix. Further therapeutic methods and compositions of the invention comprise a therapeutic amount of a bone inductive factor of the invention with a therapeutic amount of at least one of the other bone inductive factors of the invention. Additionally, the proteins according to the present invention or a combination of the proteins of the present invention may be co-administered with one or more different osteoinductive factors with which it may interact. Further, the bone inductive proteins may be combined with other agents beneficial to the treatment of the bone defect in question. Such agents include, but are not limited to various growth factors. The preparation of such physiologically acceptable protein compositions, having due regard to pH, isotonicity, stability and the like, is within the skill of the art.

In particular, BMP-1 may be used individually in a

composition. BMP-1 may also be used in combination with one or more of the other proteins of the invention. BMP-1 and BMP-2 Class I may be used in combination. BMP-1 and BMP-2 Class II may also be used in combination. BMP-1 and BMP-3 may be used in combination. Furthermore, BMP-1 may be used in combination with two or three of the other proteins of the invention. For example, BMP-1, BMP-2 Class I, and BMP-2 Class II may be combined. BMP-1 may also be combined with BMP-2 Class I, and BMP-3. Further, BMP-1 may be combined with BMP-2 Class II, and BMP-3. BMP-1, BMP-2 Class I, BMP-2 Class II, and BMP-3 may be combined.

BMP-2 Class I may be used individually in a pharmaceutical composition. BMP-2 Class I may also be used in combination with one or more of the other proteins of the invention. BMP-2 Class I may be combined with BMP-2 Class II. It may also be combined with BMP-3. Further BMP-2 Class I may be combined with BMP-2 Class II and BMP-3.

BMP-2 Class II may be used individually in pharmaceutical composition. In addition, it may be used in combination with other proteins as identified above. Further it may be used in combination with BMP-3.

BMP-3 may be used individually in a composition. It may further be used in the various combinations identified above.

The therapeutic method includes locally administering the composition as an implant or device. When administered, the therapeutic composition for use in this invention is, of course, in a pyrogen-free, physiologically acceptable form. Further, the composition may desirably be encapsulated or injected in a viscous form for delivery to the site of bone damage. Preferably, the bone growth inductive factor composition would include a matrix capable of delivering the bone inductive factor to the site of bone damage, providing a structure for the developing bone and cartilage and optimally capable of being resorbed into the body. Such matrices may be formed of other materials presently in use for other implanted medical

applications.

The choice of material is based on, for example, biocompatibility, biodegradability, mechanical properties, cosmetic appearance and interface properties. Similarly, the application of the osteoinductive factors will define the appropriate formulation. Potential matrices for the osteoinductive factors may be biodegradable and chemically defined, such as, but not limited to calcium sulfate, tricalciumphosphate, hydroxyapatite, polylactic acid, polyanhydrides; biodegradable and biologically well defined, such as bone or dermal collagen, other pure proteins or extracellular matrix components; nonbiodegradable and chemically defined, such as sintered hydroxyapatite, bioglass, aluminates, or other ceramics; or combinations of any of the above mentioned types of material, such as polylactic acid and hydroxyapatite or collagen and tricalciumphosphate. The bioceramics might also be altered in composition, such as in calcium-aluminate-phosphate and processing to alter for example, pore size, particle size, particle shape, and biodegradability.

The dosage regimen will be determined by the attending physician considering various factors which modify the action of such a growth factor, e.g. amount of bone weight desired to be formed, the site of bone damage, the condition of the damaged bone, the patient's age, sex, and diet, the severity of any infection, time of administration and other clinical factors. The dosage may vary with the type of matrix used in the reconstitution and the composition of BMP's. The addition of other known growth factors, such as IGF 1 (insulin like growth factor 1), to the final composition, may also effect the dosage. Generally, the dosage regimen should be in the range of approximately 10 to 10^6 nanograms of protein per gram of bone weight desired. Progress can be monitored by periodic assessment of bone growth and/or repair, e.g. x-rays. Such therapeutic compositions are also presently valuable for veterinary applications due to the lack of species specificity

in bone inductive factors. Particularly domestic animals and thoroughbred horses in addition to humans are desired patients for such treatment with the bone inductive factors of the present invention.

The following examples illustrate practice of the present invention in recovering and characterizing the bovine proteins and employing them to recover the human proteins, obtaining the human proteins and in expressing the proteins via recombinant techniques.

EXAMPLE I

Isolation of Bovine Bone Inductive Factor

Ground bovine bone powder (20-120 mesh, Helitrex) is prepared according to the procedures of M. R. Urist et al., Proc. Natl Acad. Sci USA, 70:3511 (1973) with elimination of some extraction steps as identified below. Ten kgs of the ground powder is demineralized in successive changes of 0.6N HCl at 4°C over a 48 hour period with vigorous stirring. The resulting suspension is extracted for 16 hours at 4°C with 50 liters of 2M CaCl₂ and 10mM ethylenediamine-tetraacetic acid [EDTA], and followed by extraction for 4 hours in 50 liters of 0.5M EDTA. The residue is washed three times with distilled water before its resuspension in 20 liters of 4M guanidine hydrochloride [GuCl], 20mM Tris (pH 7.4), 1mM N-ethylmaleimide, 1mM iodoacetamide, 1mM phenylmethylsulfonyl fluoride as described in Clin. Orthop. Rel. Res., 171: 213 (1982). After 16 to 20 hours the supernatant is removed and replaced with another 10 liters of GuCl buffer. The residue is extracted for another 24 hours.

The crude GuCl extracts are combined, concentrated approximately 20 times on a Pellicon apparatus with a 10,000 molecular weight cut-off membrane, and then dialyzed in 50mM Tris, 0.1M NaCl, 6M urea (pH7.2), the starting buffer for the first column. After extensive dialysis the protein is loaded on a 4 liter DEAE cellulose column and the unbound fractions

are collected.

The unbound fractions are concentrated and dialyzed against 50mM NaAc, 50mM NaCl (pH 4.6) in 6M urea. The unbound fractions are applied to a carboxymethyl cellulose column. Protein not bound to the column is removed by extensive washing with starting buffer, and the bone inductive factor containing material desorbed from the column by 50mM NaAc, 0.25mM NaCl, 6M urea (pH 4.6). The protein from this step elution is concentrated 20- to 40- fold, then diluted 5 times with 80mM KPO₄, 6M urea (pH6.0). The pH of the solution is adjusted to 6.0 with 500mM K₂HPO₄. The sample is applied to an hydroxylapatite column (LKB) equilibrated in 80mM KPO₄, 6M urea (pH6.0) and all unbound protein is removed by washing the column with the same buffer. Bone inductive factor activity is eluted with 100mM KPO₄ (pH7.4) and 6M urea.

The protein is concentrated approximately 10 times, and solid NaCl added to a final concentration of 0.15M. This material is applied to a heparin - Sepharose column equilibrated in 50mM KPO₄, 150mM NaCl, 6M urea (pH7.4). After extensive washing of the column with starting buffer, a protein with bone inductive factor activity is eluted by 50mM KPO₄, 700mM NaCl, 6M urea (pH7.4). This fraction is concentrated to a minimum volume, and 0.4ml aliquots are applied to Superose 6 and Superose 12 columns connected in series, equilibrated with 4M GuCl, 20mM Tris (pH7.2) and the columns developed at a flow rate of 0.25ml/min. The protein demonstrating bone inductive factor activity has a relative migration corresponding to approximately 30,000 dalton protein.

The above fractions are pooled, dialyzed against 50mM NaAc, 6M urea (pH4.6), and applied to a Pharmacia MonoS HR column. The column is developed with a gradient to 1.0M NaCl, 50mM NaAc, 6M urea (pH4.6). Active fractions are pooled and brought to pH3.0 with 10% trifluoroacetic acid (TFA). The material is applied to a 0.46 x 25cm Vydac C4 column in 0.1% TFA and the column developed with a gradient to 90% acetonitrile, 0.1% TFA

(31.5% acetonitrile, 0.1% TFA to 49.5% acetonitrile, 0.1% TFA in 60 minutes at 1ml per minute). Active material is eluted at approximately 40-44% acetonitrile. Aliquots of the appropriate fractions are iodinated by one of the following methods: P. J. McConahey et al, Int. Arch. Allergy, 29:185-189 (1966); A. E. Bolton et al, Biochem J., 133:529 (1973); and D. F. Bowen-Pope, J. Biol. Chem., 237:5161 (1982). The iodinated proteins present in these fractions are analyzed by SDS gel electrophoresis and urea Triton X 100 isoelectric focusing. At this stage, the bone inductive factor is estimated to be approximately 10-50% pure.

EXAMPLE II

Characterization of Bovine Bone Inductive Factor

A. Molecular Weight

Approximately 20ug protein from Example I is lyophilized and redissolved in 1X SDS sample buffer. After 15 minutes of heating at 37°C, the sample is applied to a 15% SDS polyacrylamide gel and then electrophoresed with cooling. The molecular weight is determined relative to prestained molecular weight standards (Bethesda Research Labs). Immediately after completion, the gel lane containing bone inductive factor is sliced into 0.3cm pieces. Each piece is mashed and 1.4ml of 0.1% SDS is added. The samples are shaken gently overnight at room temperature to elute the protein. Each gel slice is desalted to prevent interference in the biological assay. The supernatant from each sample is acidified to pH 3.0 with 10% TFA, filtered through a 0.45 micron membrane and loaded on a 0.46cm x 5cm C4 Vydac column developed with a gradient of 0.1% TFA to 0.1% TFA, 90% CH₃CN. The appropriate bone inductive factor - containing fractions are pooled and reconstituted with 20mg rat matrix. In this gel system, the majority of bone inductive factor fractions have the mobility of a protein having a molecular weight of approximately 28,000 - 30,000 daltons.

B. Isoelectric Focusing

The isoelectric point of bone inductive factor activity is determined in a denaturing isoelectric focusing system. The Triton X100 urea gel system (Hoeffer Scientific) is modified as follows: 1) 40% of the ampholytes used are Servalyte 3/10; 60% are Servalyte 7-9. 2) The catholyte used is 40mM NaOH. Approximately 20ug of protein from Example I is lyophilized, dissolved in sample buffer and applied to the isoelectrofocusing gel. The gel is run at 20 watts, 10°C for approximately 3 hours. At completion the lane containing bone inductive factor is sliced into 0.5 cm slices. Each piece is mashed in 1.0ml 6M urea, 5mM Tris (pH 7.8) and the samples agitated at room temperature. The samples are acidified, filtered, desalted and assayed as described above. The major portion of activity as determined in the assay described in Example III migrates in a manner consistent with a pI of 8.8 - 9.2.

C. Subunit Characterization

The subunit composition of bone inductive factor is also determined. Pure bone inductive factor is isolated from a preparative 15% SDS gel as described above. A portion of the sample is then reduced with 5mM DTT in sample buffer and re-electrophoresed on a 15% SDS gel. The approximately 30kd protein yields two major bands at approximately 20kd and 18kd, as well as a minor band at 30kd. The broadness of the two bands indicates heterogeneity caused most probably by glycosylation, other post translational modification, proteolytic degradation or carbamylation.

EXAMPLE III

Biological Activity of Bone Inductive Factor

A rat bone formation assay according to the general procedure of Sampath and Reddi, Proc. Natl. Acad. Sci. U.S.A.,

80:6591-6595 (1983) is used to evaluate the osteogenic activity of the bovine bone inductive factor of the present invention obtained in Example I. This assay can also be used to evaluate bone inductive factors of other species. The ethanol precipitation step is replaced by dialyzing the fraction to be assayed against water. The solution or suspension is then redissolved in a volatile solvent, e.g. 0.1 - 0.2 % TFA, and the resulting solution added to 20mg of rat matrix. This material is frozen and lyophilized and the resulting powder enclosed in #5 gelatin capsules. The capsules are implanted subcutaneously in the abdominal thoracic area of 21 - 49 day old male long Evans rats. The implants are removed after 7 - 14 days. Half of each implant is used for alkaline phosphatase analysis [See, A. H. Reddi et al., Proc. Natl Acad Sci., 69:1601 (1972)] and half is fixed and processed for histological analysis. Routinely, 1um glycolmethacrylate sections are stained with Von Kossa and acid fuschin to detect new bone mineral. Alkaline phosphatase, an enzyme produced by chondroblasts and osteoblasts in the process of matrix formation, is also measured. New cartilage and bone formation often correlates with alkaline phosphatase levels. Table I below illustrates the dose response of the rat matrix samples including a control not treated with bone inductive factor.

TABLE 1

| <u>Protein*</u> <u>Implanted ug</u> | <u>Cartilage</u> | <u>Alk. Phos.u/l</u> |
|--|------------------|----------------------|
| 7.5 | 2 | Not done |
| 2.5 | 3 | 445.7 |
| 0.83 | 3 | 77.4 |
| 0.28 | 0 | 32.5 |
| 0.00 | 0 | 31.0 |

*At this stage the bone inductive factor is approximately 10-15% pure.

The bone or cartilage formed is physically confined to the space occupied by the matrix. Samples are also analyzed by SDS gel electrophoresis and isoelectric focusing as described

above, followed by autoradiography. Analysis reveals a correlation of activity with protein bands at 28 - 30kd and a pI 9.0. An extinction coefficient of 1 OD/mg-cm is used as an estimate for protein and approximating the purity of bone inductive factor in a particular fraction. In the in vivo rat bone formation assays on dilutions as described above, the protein is active in vivo at 10 to 200ng protein/gram bone to probably greater than 1ug protein/gram bone.

EXAMPLE IV

Bovine Bone Inductive Factor Protein Composition

The protein composition of Example IIA of molecular weight 28 - 30kd is reduced as described in Example IIC and digested with trypsin. Eight tryptic fragments are isolated by standard procedures having the following amino acid sequences:

Fragment 1: A A F L G D I A L D E E D L G

Fragment 2: A F Q V Q Q A A D L

Fragment 3: N Y Q D M V V E G

Fragment 4: S T P A Q D V S R

Fragment 5: N Q E A L R

Fragment 6: L S E P D P S H T L E E

Fragment 7: F D A Y Y

Fragment 8: L K P S N ? A T I Q S I V E

A less highly purified preparation of protein from bovine bone is prepared according to a purification scheme similar to that described in Example I. The purification basically varies from that previously described by omission of the DE-52 column, the CM cellulose column and the mono S column, as well as a reversal in the order of the hydroxylapatite and heparin sepharose columns. Briefly, the concentrated crude 4 M extract is brought to 85% final concentration of ethanol at 4 degrees. The mixture is then centrifuged, and the precipitate redissolved in 50 mM Tris, 0.15 M NaCl, 6.0 M urea. This material is then fractionated on Heparin Sepharose as described. The Heparin bound material

is fractionated on hydroxyapatite as described. The active fractions are pooled, concentrated, and fractionated on a high resolution gel filtration (TSK 30000 in 6 M guanidinium chloride, 50 mM Tris, pH 7.2). The active fractions are pooled, dialyzed against 0.1% TFA, and then fractionated on a C4 Vydac reverse phase column as described. The preparation is reduced and electrophoresed on an acrylamide gel. The protein corresponding to the 18K band is eluted and digested with trypsin. Tryptic fragments are isolated having the following amino acid sequences:

Fragment 9: S L K P S N H A T I Q S ? V

Fragment 10: S F D A Y Y C S ? A

Fragment 11: V Y P N M T V E S C A

Fragment 12: V D F A D I ? W

Tryptic Fragments 7 and 8 are noted to be substantially the same as Fragments 10 and 9, respectively.

A. bBMP-1

Probes consisting of pools of oligonucleotides (or unique oligonucleotides) are designed according to the method of R. Lathe, J. Mol. Biol., 183 (1):1-12 (1985) and synthesized on an automated DNA synthesizer. One probe consists of a relatively long (32 nucleotides) "guessmer" [See J. J. Toole et al, Nature, 312:342-347 (1984)] of the following nucleotide sequence:

TCCTCATCCAGGGCAATGTCGCCCAAGGAGGC

Because the genetic code is degenerate (more than one codon can code for the same amino acid), the number of oligonucleotides in a probe pool is reduced based on the frequency of codon usage in eukaryotes, the relative stability of G:T base pairs, and the relative infrequency of the dinucleotide CpG in eukaryotic coding sequences [see Toole et al., *supra.*]. The second set of probes consists of shorter oligonucleotides (17 nucleotides in length) which contain all possible sequences that could encode the amino acids. The second set of probes has the following sequences:

(a) A [A/G] [A/G] TC [T/C] TC [T/C] TC [A/G] TC [T/C] AA

(b) A [A/G] [A/G] TC [T/C] TC [T/C] TC [A/G] TCNAG

Bracketed nucleotides are alternatives. "N" means either A, T, C or G.

In both cases the regions of the amino acid sequence used for probe design are chosen by avoiding highly degenerate codons where possible. The oligonucleotides are synthesized on an automated DNA synthesizer; the probes are then radioactively labeled with polynucleotide kinase and ^{32}P -ATP.

These two sets of probes are used to screen a bovine genomic recombinant library. The library is constructed as follows: Bovine liver DNA is partially digested with the restriction endonuclease enzyme Sau 3A and sedimented through a sucrose gradient. Size fractionated DNA in the range of 15-30kb is then ligated to the bacteriophage Bam HI vector EMBL3 [Frischauf et al, J. Mol. Biol., 170:827-842 (1983)]. The library is plated at 8000 recombinants per plate. Duplicate nitrocellulose replicas of the plaques are made and amplified according to a modification of the procedure of Woo et al, Proc. Natl. Acad. Sci. USA, 75:3688-91 (1978).

The 32 mer probe is kinased with ^{32}P -gamma-ATP and hybridized to one set of filters in 5X SSC, 0.1% SDS, 5X Denhardts, 100ug/ml salmon sperm DNA at 45 degrees C and washed with 5X SSC, 0.1% SDS at 45 degrees C. The 17 mer probes are kinased and hybridized to the other set of filters in 3M tetramethylammonium chloride (TMAC), 0.1M sodium phosphate pH6.5, 1mM EDTA, 5X Denhardts, 0.6% SDS, 100ug/ml salmon sperm DNA at 48 degrees C, and washed in 3M TMAC, 50mM Tris pH8.0 at 50 degrees C. These conditions minimize the detection of mismatches to the 17 mer probe pool [see, Wood et al, Proc. Natl. Acad. Sci. U.S.A., 82:1585-1588 (1985)]. 400,000 recombinants are screened by this procedure and one duplicate positive is plaque purified. DNA is isolated from a plate lysate of this recombinant bacteriophage designated lambda bP-50. bP-50 was deposited December 16, 1986 with the American

Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland USA (hereinafter the "ATCC") under accession number 40295. This deposit as well as the other deposits contained herein meets the requirements of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and Regulations thereunder. This bp-50 clone encodes at least a portion of the bovine bone growth factor designated bBMP-1.

The oligonucleotide hybridizing region of this bBMP-1 clone is localized to an approximately 800bp Eco RI fragment which is subcloned into M13 and sequenced by standard techniques. The partial DNA sequence and derived amino acid sequence of lambda bP-50 are shown below in Table II. The amino acid sequences corresponding to the tryptic fragments isolated from the bovine bone 28 to 30kd material are underlined in Table II. The first underlined portion of the sequence corresponds to tryptic Fragment 1 above from which the oligonucleotide probes are designed. The second underlined portion corresponds to tryptic Fragment 2 above. The predicted amino acid sequence indicates that tryptic Fragment 2 is preceded by a basic residue (R) as expected considering the specificity of trypsin. The nucleic acid sequence preceding the couplet CT at nucleotide positions #292-293 in Table II is presumed to be an intron (noncoding sequence) based on the presence of a consensus acceptor sequence (i.e., a pyrimidine rich tract, TCTCTCTCC, followed by AG) and the lack of a basic residue in the appropriate position of the derived amino acid sequence. This bBMP-1 genomic sequence appears in Table II. The presumptive bBMP-1 peptide sequence from this genomic clone is 37 amino acids in length and is encoded by the DNA sequence from nucleotide #294 through #404 in Table II.

TABLE II

| | | | | |
|--|------------|--|-----|-----|
| 280 | 290 | (1) | 308 | 323 |
| CCTTGCCCTCT | TCTCTCTCCA | GCT GGC TTC CTT GGG GAC ATC GCC CTG GAC GAG GAG | | |
| | | <u>Ala Phe Leu Gly Asp Ile Ala Leu Asp Glu Glu</u> | | |
| | 338 | 353 | 368 | |
| GAC TTG AGG GGC TTC CAA GTG CAG CAG GCT GCG GAC CTC AGA CAG CGT GCA ACC | | | | |
| <u>Asp Leu Arg Ala Phe Gln Val Gln Gln Ala Ala Asp Leu Arg Gln Arg Ala Thr</u> | | | | |
| 383: | 398 | (37) | 414 | 424 |
| CGC AGG TCT TCC ATC AAA GCT GCA GGTACACTGG GTACAGGCCA | | | | |
| Arg Arg Ser Ser Ile Lys Ala Ala | | | | |

B. bBMP-2

Two probes consisting of pools of oligonucleotides are designed on the basis of the amino acid sequence of Fragment 3 and synthesized on an automated DNA synthesizer as described above.

Probe #1: A C N A C C A T [A/G] T C [T/C] T G [A/G] A T

Probe #2: C A [A/G] G A [T/C] A T G G T N G T N G A

These probes are radioactively labeled and employed to screen the bovine genomic library constructed as described in part A except that the vector is lambda J1 Bam HI arms [Mullins et al Nature 308: 856-858 (1984).] The radioactively labelled 17-mer Probe #1 is hybridized to the set of filters according to the method for the 17 mer probe described in part A.

400,000 recombinants are screened by the procedure described above in Part A. One duplicate positive is plaque purified and the DNA is isolated from a plate lysate of the recombinant bacteriophage designated lambda bP-21. Bacteriophage bP-21 was deposited with the ATCC under accession number ATCC 40310 on March 6, 1987. The bP-21 clone encodes the bovine growth factor designated bBMP-2.

The oligonucleotide hybridizing region of this bBMP-2 clone is localized to an approximately 1.2 kb Sac I restriction fragment which is subcloned into M13 and sequenced by standard techniques. The partial DNA sequence and derived amino acid sequence of this Sac I fragment and the contiguous Hind III-

Sac I restriction fragment of bP-21 are shown below in Table III. The bBMP-2 peptide sequence from this clone is 129 amino acids in length and is encoded by the DNA sequence from nucleotide #1 through nucleotide #387. The amino acid sequence corresponding to the tryptic fragment isolated from the bovine bone 28 to 30kd material is underlined in Table III. The underlined portion of the sequence corresponds to tryptic Fragment 3 above from which the oligonucleotide probes for bBMP-2 are designed. The predicted amino acid sequence indicates that tryptic Fragment 3 is preceded by a basic

residue (K) as expected considering the specificity of trypsin. The arginine residue encoded by the CGT triplet is presumed to be the carboxy-terminus of the protein based on the presence of a stop codon (TAG) adjacent to it.

TABLE III

| | | | |
|---|-----|-------|-----|
| (1) | 15 | 30 | 45 |
| GGC CAC GAT GGG AAA GGA CAC CCT CTC CAC AGA AGA GAA AAG CGG | | | |
| G H D G K G H P L H R R E K R | | | |
| CAA GCA AAA CAC AAA CAG CGG AAA CGC CTC AAG TCC AGC TGT AAG | 60 | 75 | 90 |
| Q A K H K Q R K R L K S S C K | | | |
| AGA CAC CCT TTA TAT GTG GAC TTC AGT GAT GTG GGG TGG AAT GAC | 105 | 120 | 135 |
| R H P L Y V D F S D V G W N D | | | |
| TGG ATC GTT GCA CCG CCG GGG TAT CAT GCC TTT TAC TGC CAT GGG | 150 | 165 | 180 |
| W I V A P P G Y H A F Y C H G | | | |
| GAG TGC CCT TTT CCC CTG GCC GAT CAC CTT AAC TCC ACG AAT CAT | 195 | 210 | 225 |
| E C P F P L A D H L N S T N H | | | |
| GCC ATT CTC CAA ACT CTG GTC AAC TCA GTT AAC TCT AAG ATT CCC | 240 | 255 | 270 |
| A I V Q T L V N S V N S K I P | | | |
| AAG GCA TGC TGT GTC CCA ACA GAG CTC AGC GCC ATC TCC ATG CTG | 385 | 300 | 315 |
| K A C C V P T E L S A I S M L | | | |
| TAC CTT GAT GAG AAT GAG AAG GTG GTA TTA AAG AAC TAT CAG GAC | 330 | 345 | 360 |
| Y L D E N E K V V L K N Y Q D | | | |
| ATG GTT GTC GAG GGT TGT GGG TGT CGT TAGCACAGCA AAATAAAATA | 375 | (129) | 397 |
| M V V E G C G C R | | | 407 |
| 417 | 427 | 437 | 447 |
| TAAATATATA TATATATATA TTAGAAAAAC AGCAAAAAAA TCAAGTTGAC | | | 457 |
| 467 | 477 | 487 | 497 |
| ACTTTAATAT TTCCCAATGA AGACTTTATT TATGGAATGG AATGGAGAAA | | | 507 |
| 517 | 527 | 537 | 547 |
| AAGAAAAACA CAGCTATTTT GAAAACTATA TTTATATCTA CCGAAAAGAA | | | 557 |
| 567 | 577 | 587 | |
| GTTGGGAAAA CAAATATTTT AATCAGAGAA TTATT | | | |

C. bBMP-3

Probes consisting of pools of oligonucleotides are designed on the basis of the amino acid sequences of the tryptic Fragments 9 (Probe #3), 10 (Probe #2), and 11 (Probe #1), and synthesized on an automated DNA synthesizer.

Probe #1: A C N G T C A T [A/G] T T N G G [A/G] T A

Probe #2: C A [A/G] T A [A/G] T A N G C [A/G] T C [A/G] A A

Probe #3: T G [A/G/T] A T N G T N G C [A/G] T G [A/G] T T

A recombinant bovine genomic library constructed in EMBL3 is screened by the TMAC hybridization procedure detailed above in part A. 400,000 recombinants are screened in duplicate with Probe #1 which has been labeled with ^{32}P . All recombinants which hybridized to this probe are replated for secondaries. Triplicate nitrocellulose replicas are made of the secondary plates, and amplified as described. The three sets of filters are hybridized to Probes #1, #2 and #3, again under TMAC conditions. One clone, lambda bP-819, hybridizes to all three probes and is plaque purified and DNA is isolated from a plate lysate. Bacteriophage lambda bP-819 was deposited with the ATCC on June 16, 1987 under accession number 40344. This bP-819 clone encodes the bovine bone growth factor designated bBMP-3.

The region of bP-819 which hybridizes to Probe #2 is localized and sequenced. The partial DNA and derived amino acid sequences of this region are shown in Table IVA. The amino acid sequences corresponding to tryptic Fragments 10 and 12 are underlined. The first underlined sequence corresponds to Fragment 12 while the second corresponds to Fragment 10. This region of bP-819, therefore, which hybridizes to Probe #2 encodes at least 111 amino acids. This amino acid sequence is encoded by the DNA sequence from nucleotide #414 through #746.

TABLE IV. A.

| | | | | | |
|---|------------|-----------|------------|-----------------------------|-----------------------------|
| 383 | 393 | 403 | 413 | (1) | 428 |
| GAGGAGGAAG | OGGTCTAOGG | GGGTCTTCT | GOCTCTGCAG | AAC AAT GAG CTT OCT GGG GCA | Asn Asn Glu Leu Pro Gly Ala |
| 443 | 458 | 473 | 488 | | |
| GAA TAT CAG TAC AAG GAG GAT GAA GTA TGG GAG GAG AGG AAG CCT TAC AAG ACT | | | | | |
| Glu Tyr Gln Tyr Lys Glu Asp Glu Val Trp Glu Glu Arg Lys Pro Tyr Lys Thr | | | | | |
| 503 | 518 | 533 | | | |
| CTT CAG ACT CAG CCC CCT GAT AAG AGT AAG AAC AAA AAG AAA CAG AGG AAG GGA | | | | | |
| Leu Gln Thr Gln Pro Pro Asp Lys Ser Lys Asn Lys Lys Lys Gln Arg Lys Gly | | | | | |
| 548 | 563 | 578 | 593 | | |
| CCT CAG CAG AAG AGT CAG ACG CTC CAG TTT GAT GAA CAG ACC CTG AAG AAG GCA | | | | | |
| Pro Gln Gln Lys Ser Gln Thr Leu Gln Phe Asp Glu Gln Thr Leu Lys Lys Ala | | | | | |
| 608 | 623 | 638 | | | |
| AGA AGA AAG CAA TGG ATT GAA CCC CGG AAT TGT GCC AGA CGG TAC CTT AAA GTG | | | | | |
| Arg Arg Lys Gln Trp Ile Glu Pro Arg Asn Cys Ala Arg Arg Tyr Leu Lys Val | | | | | |
| 653 | 668 | 683 | 698 | | |
| GAC TTC GCA GAT ATT GGC TGG AGC GAA TGG ATT ATT TOC CCC AAG TOC TTC GAT | | | | | |
| Asp Phe Ala Asp Ile Gly Trp Ser Glu Trp Ile Ile Ser Pro Lys Ser Phe Asp | | | | | |
| 713 | 728 | 743 (111) | 756 | | |
| GCC TAT TAC TGC TOC GGA GCG TGC CAG TTC CCC ATG CCA AAG GTAGCCATTG | | | | | |
| Ala Tyr Tyr Cys Ser Gly Ala Cys Gln Phe Pro MET Pro Lys | | | | | |
| 766 | 776 | 786 | | | |
| TTTTTGTCC | TGTCCTTCCC | ATTTCATAG | | | |

The region of bP-819 which hybridizes to Probe #1 and #3 is localized and sequenced. The partial DNA and derived amino acid sequences of this region are shown in Table IVB. The amino acid sequences corresponding to tryptic Fragments 9 and 11 are underlined. The first underlined sequence corresponds to Fragment 9 while the second underlined sequence corresponds to Fragment 11. The peptide sequence of this region of bP-819 which hybridizes to Probe #1 and #3 is 64 amino acids in length encoded by nucleotide #305 through #493 of Table IVB. The arginine residue encoded by the AGA triplet is presumed to be the carboxy-terminus of the protein based on the presence of a stop codon (TAA) adjacent to it. The nucleic acid sequence preceding the couplet TC (positions 305-306) is presumed to be an intron (non-coding sequence) based on the presence of a consensus acceptor sequence (i.e. a pyrimidine-rich stretch, TTCTCCCTTTTCGTTCT, followed by AG) and the presence of a stop rather than a basic residue in the appropriate position of the derived amino acid sequence.

bBMP-3 is therefore characterized by the DNA and amino acid sequence of Table IV A and Table IV B. The peptide sequence of this clone is 175 amino acids in length and is encoded by the DNA sequence from nucleotide #414 through nucleotide #746 of Table IV A and nucleotide #305 through nucleotide #493 of Table IV B.

TABLE IV. B.

| | | | | |
|--|---|-------------|--|-----|
| 284 | 294 | 304 | (112) | 319 |
| CTAACCTGGT | TTCTCCCTTT | TOGTTCCCTAG | TCT TTG AAG CCA TCA AAT CAC GCT ACC | |
| | | | <u>Ser Leu Lys Pro Ser Asn His Ala Thr</u> | |
| 334 | 349 | 364 | 379 | |
| ATC CAG AGT ATA GTG AGA GCT GTG GGG GTC GTC CCT GGA ATC CCC GAG CCT TGC | | | | |
| <u>Ile Gln Ser Ile Val</u> | Arg Ala Val Gly Val Val Pro Gly Ile Pro Glu Pro Cys | | | |
| 394 | 409 | 424 | 439 | |
| TGT GTG CCA GAA AAG ATG TCC TCA CTC AGC ATC TTA TTC TTT GAT GAA AAC AAG | | | | |
| Cys Val Pro Glu Lys MET Ser Ser Leu Ser Ile Leu Phe Phe Asp Glu Asn Lys | | | | |
| 454 | 469 | 484 | (175) | |
| AAT GTG GTA CTT AAA GTA TAT CCA AAC ATG ACA GTA GAG TCT TGT GCT TGC AGA | | | | |
| Asn Val Val Leu Lys <u>Val Tyr Pro Asn MET Thr Val Glu Ser Cys Ala Cys Arg</u> | | | | |
| 503 | 513 | 523 | 533 | |
| TAACCTGGTG | AAGAACTCAT | CTGGATGCTT | AACTCAATCG | |

EXAMPLE VHuman Bone Inductive FactorsA. hBMP-1

Because the bovine and human bone growth factor genes are presumed to be significantly homologous, the bovine bBMP-1 DNA sequence of Table II (or portions thereof) is used as a probe to screen a human genomic library. The 800bp EcoRI fragment of the bovine genomic clone is labeled with ^{32}P by nick-translation. A human genomic library (Toole et al., supra) is plated on 20 plates at 40,000 recombinants per plate. Duplicate nitrocellulose filter replicas are made of each plate and hybridized to the nick-translated probe in 5 X SSC, 5 X Denhardt's, 100ug/ml denatured salmon sperm DNA, 0.1% SDS (the standard hybridization solution) at 50 degrees centigrade for approximately 14 hours. The filters are then washed in 1 X SSC, 0.1% SDS at 50 degrees centigrade and subjected to autoradiography. Five duplicate positives are isolated and plaque purified. DNA is obtained from a plate lysate of one of these recombinant bacteriophage, designated LP-H1. LP-H1 was deposited with the ATCC on March 6, 1987 under accession number 40311. This clone encodes at least a portion of the human genomic bone growth factor called hBMP-1. The hybridizing region of LP-H1 is localized to a 2.5kb XbaI/HindIII restriction fragment.

The partial DNA sequence and derived amino acid sequence of lambda LP-H1 are shown below in Table V. The peptide sequence from this clone is 37 amino acids in length and is encoded by the DNA sequence from nucleotide #3440 through nucleotide #3550. The coding sequence of Table V is flanked by approximately 28 nucleotides (a presumptive 5' noncoding sequence) as well as approximately 19 nucleotides (a presumptive 3' noncoding sequence). A comparison of the bBMP-1 sequence of Table II with the hBMP-1 genomic sequence of Table V indicates the significant homology between the two.

Because the size of coding regions and the positions

of noncoding regions is generally conserved in homologous genes of different species, the locations of the coding and noncoding regions of the bone inductive factor genes may be identified. Regions of homology between the two species' genes, flanked by RNA processing signals at homologous sites, indicate a coding region.

TABLE V

| | | | | |
|---|------------|------------|-------------------------------------|------|
| 3419 | 3429 | 3439 | (1) | 3454 |
| CAGCCCTGGC | TTCTTCITTT | CICITTAGCT | GCC TTT CTT GGG GAC ATT GCC CTG GAC | |
| | | | Ala Phe Leu Gly Asp Ile Ala Leu Asp | |
| 3469 | 3484 | 3499 | 3514 | |
| GAA GAG GAC CTG AGG GCC TTC CAG GTA CAG CAG GCT GTG GAT CTC AGA OGG CAC | | | | |
| Glu Glu Asp Leu Arg Ala Phe Gln Val Gln Gln Ala Val Asp Leu Arg Arg His | | | | |
| 3529 | 3544 | (37) | 3560 | 3570 |
| ACA GCT CGT AAG TCC TCC ATC AAA GCT GCA GGTAAGCCGG GTGCCAATGG | | | | |
| Thr Ala Arg Lys Ser Ser Ile Lys Ala Ala | | | | |

A probe specific for the human coding sequence given in Table V is used to identify a human cell line or tissue which synthesizes bone inductive factor. The probe is made according to the following method. Two oligonucleotides having the following sequences:

(a) GGGAATTCTGCCTTTCTTGGGGACATTGCCCTGGACGAAGAGGACCTGAG

(b) CGGGATCCGTCTGAGATCCACAGCCTGCTGTACCTGGAAGGCCCTCAGG

are synthesized on an automated synthesizer, annealed, extended using the Klenow fragment of E. coli DNA polymerase I, digested with the restriction enzymes Eco RI and Bam HI, and inserted into an M13 vector. A single-stranded ³²P-labeled probe is then from template preparation of this subclone by standard techniques. Polyadenylated RNAs from various cell and tissue sources are electrophoresed on formaldehyde-agarose gels and transferred to nitrocellulose by the method of Toole et al., supra. The probe is then hybridized to the nitrocellulose blot in 50% formamide, 5 X SSC, 0.1% SDS, 40 mM sodium phosphate pH 6.5, 100 ug/ml denatured salmon sperm DNA, and 5 mM vanadyl ribonucleosides at 42° C overnight and washed at 65° C in 0.2 X SSC, 0.1% SDS. Following autoradiography, the lane containing RNA from the human osteosarcoma cell line U-2 OS contains hybridizing bands corresponding to RNA species of approximately 4.3 and 3.0 kb.

cDNA is synthesized from U-2 OS polyadenylated RNA and cloned into lambda gt10 by established techniques (Toole et al., supra). 20,000 recombinants from this library are plated on each of 50 plates. Duplicate nitrocellulose replicas are made of the plates. The above described oligonucleotides are kinased with ³²P-gamma-ATP and hybridized to the two sets of replicas at 55° centigrade in standard hybridization solution overnight. The filters are then washed in 1 X SSC, 0.1% SDS at 55° centigrade and subjected to autoradiography. One duplicate positive, designated lambda U2OS-1, is plaque purified. Lambda U2OS-1 was deposited with the ATCC on June 16, 1987 under accession number 40343.

The entire nucleotide sequence and derived amino acid sequence of the insert of lambda U20S-1 is given in Table VI. This cDNA clone encodes a Met followed by a hydrophobic leader sequence characteristic of a secreted protein, and contains a stop codon at nucleotide positions 2226 - 2228. This clone contains an open reading frame of 2190bp, encoding a protein of 730 amino acids with a molecular weight of 83kd based on this amino acid sequence. The clone contains sequence identical to the coding region given in Table V. This protein is contemplated to represent a primary translation product which is cleaved upon secretion to produce the hBMP-1 protein. This clone is therefore a cDNA for hBMP-1 corresponding to human gene fragment contained in the genomic hBMP-1 sequence lambda LP-H1. It is noted that amino acids #550 to #590 of BMP-1 are homologous to epidermal growth factor and the "growth factor" domains of Protein C, Factor X and Factor IX.

TABLE VI

| | | | | |
|---|-----------|-----------|-----------|-----------------------------|
| 10 | 20 | 30 | (1) | 50 |
| CTAGAGGCG | CTTCCTCGC | CGCGGCGCG | CCAGC ATG | CCC GGC GTG GGC CGC CTG CCG |
| | | | MET | Pro Gly Val Ala Arg Leu Pro |
| 65 | 80 | 95 | 110 | |
| CTG CTG CTC GGG CTG CTG CTG CTC CCG CGT CCC GGC CGG CCG CTG GAC TTG GGC | | | | |
| Leu Leu Leu Gly Leu Leu Leu Leu Pro Arg Pro Gly Arg Pro Leu Asp Leu Ala | | | | |
| 125 | 140 | 155 | | |
| GAC TAC ACC TAT GAC CTG GCG GAG GAG GAC GAC TCG GAG CCC CTC AAC TAC AAA | | | | |
| Asp Tyr Thr Tyr Asp Leu Ala Glu Glu Asp Asp Ser Glu Pro Leu Asn Tyr Lys | | | | |
| 170 | 185 | 200 | 215 | |
| GAC CCC TGC AAG GCG GCT GCG TTT CTT GGC GAC ATT GCC CTG GAC GAA GAG GAC | | | | |
| Asp Pro Cys Lys Ala Ala Ala Phe Leu Gly Asp Ile Ala Leu Asp Glu Glu Asp | | | | |
| 230 | 245 | 260 | 275 | |
| CTG AGG GCG TTC CAG GTA CAG CAG GCT GTG GAT CTC AGA CGG CAC ACA GCT CGT | | | | |
| Leu Arg Ala Phe Gln Val Gln Gln Ala Val Asp Leu Arg Arg His Thr Ala Arg | | | | |
| 290 | 305 | 320 | | |
| AAG TCC TCC ATC AAA GCT GCA GTT CCA GGA AAC ACT TCT ACC CCC AGC TGC CAG | | | | |
| Lys Ser Ser Ile Lys Ala Ala Val Pro Gly Asn Thr Ser Thr Pro Ser Cys Gln | | | | |
| 335 | 350 | 365 | 380 | |
| AGC ACC AAC GGG CAG CCT CAG AGG GGA GCC TGT GGG AGA TGG AGA GGT AGA TCC | | | | |
| Ser Thr Asn Gly Gln Pro Gln Arg Gly Ala Cys Gly Arg Trp Arg Gly Arg Ser | | | | |
| 395 | 410 | 425 | | |
| CGT AGC CGG CGG GCG GCG ACG TCC CGA CCA GAG CGT GTG TGG CCC GAT GGG GTC | | | | |
| Arg Ser Arg Arg Ala Ala Thr Ser Arg Pro Glu Arg Val Trp Pro Asp Gly Val | | | | |
| 440 | 455 | 470 | 485 | |
| ATC CCC TTT GTC ATT GGG GGA AAC TTC ACT GGT AGC CAG AGG GCA GTC TTC CCG | | | | |
| Ile Pro Phe Val Ile Gly Gly Asn Phe Thr Gly Ser Gln Arg Ala Val Phe Arg | | | | |
| 500 | 515 | 530 | 545 | |
| CAG GCG ATG AGG CAC TGG GAG AAG CAC ACC TGT GTC ACC TTC CTG GAG CGC ACT | | | | |
| Gln Ala MET Arg His Trp Glu Lys His Thr Cys Val Thr Phe Leu Glu Arg Thr | | | | |
| 560 | 575 | 590 | | |
| GAC GAG GAC AGC TAT ATT GTG TTC ACC TAT CGA CCT TGC GGG TGC TGC TCC TAC | | | | |
| Asp Glu Asp Ser Tyr Ile Val Phe Thr Tyr Arg Pro Cys Gly Cys Cys Ser Tyr | | | | |
| 605 | 620 | 635 | 650 | |
| GTG GGT CGC CGC GGC GCG GCG CCC CAG GCC ATC TCC ATC GGC AAG AAC TGT GAC | | | | |
| Val Gly Arg Arg Gly Gly Gly Pro Gln Ala Ile Ser Ile Gly Lys Asn Cys Asp | | | | |

665 680 695
 AAG TTC GGC ATT GTG GTC CAC GAG CTG GGC CAC GTC GTC GGC TTC TGG CAC GAA
 Lys Phe Gly Ile Val Val His Glu Leu Gly His Val Val Gly Phe Trp His Glu

710 725 740 755
 CAC ACT CGG CCA GAC CGG GAC OGC CAC GTT TCC ATC GTT OGT GAG AAC ATC CAG
 His Thr Arg Pro Asp Arg Asp Arg His Val Ser Ile Val Arg Glu Asn Ile Gln

770 785 800 815
 CCA GGG CAG GAG TAT AAC TTC CTG AAG ATG GAG CCT CAG GAG GTG GAG TCC CTG
 Pro Gly Gln Glu Tyr Asn Phe Leu Lys MET Glu Pro Gln Glu Val Glu Ser Leu

830 845 860
 GGG GAG ACC TAT GAC TTC GAC AGC ATC ATG CAT TAC GCT OGG AAC ACA TTC TCC
 Gly Glu Thr Tyr Asp Phe Asp Ser Ile MET His Tyr Ala Arg Asn Thr Phe Ser

875 890 905 920
 AGG GGC ATC TTC CTG GAT ACC ATT GTC CCC AAG TAT GAG GTG AAC GGG GTG AAA
 Arg Gly Ile Phe Leu Asp Thr Ile Val Pro Lys Tyr Glu Val Asn Gly Val Lys

935 950 965
 CCT CCC ATT GGC CAA AGG ACA OGC CTC AGC AAG GGG GAC ATT GCC CAA GCC OGC
 Pro Pro Ile Gly Gln Arg Thr Arg Leu Ser Lys Gly Asp Ile Ala Gln Ala Arg

980 995 1010 1025
 AAG CTT TAC AAG TGC CCA GCC TGT GGA GAG ACC CTG CAA GAC AGC ACA GGC AAC
 Lys Leu Tyr Lys Cys Pro Ala Cys Gly Glu Thr Leu Gln Asp Ser Thr Gly Asn

1040 1055 1070 1085
 TTC TCC TCC CCT GAA TAC CCC AAT GGC TAC TCT GCT CAC ATG CAC TGC GTG TGG
 Phe Ser Ser Pro Glu Tyr Pro Asn Gly Tyr Ser Ala His MET His Cys Val Trp

1100 1115 1130
 OGC ATC TCT GTC ACA CCC GGG GAG AAG ATC ATC CTG AAC TTC ACG TCC CTG GAC
 Arg Ile Ser Val Thr Pro Gly Glu Lys Ile Ile Leu Asn Phe Thr Ser Leu Asp

1145 1160 1175 1190
 CTG TAC OGC AGC OGC CTG TGC TGG TAC GAC TAT GTG GAG GTC OGA GAT GGC TTC
 Leu Tyr Arg Ser Arg Leu Cys Trp Tyr Asp Tyr Val Glu Val Arg Asp Gly Phe

1205 1220 1235
 TGG AGG AAG GCG CCC CTC OGA GGC OGC TTC TGC GGG TCC AAA CTC CCT GAG CCT
 Trp Arg Lys Ala Pro Leu Arg Gly Arg Phe Cys Gly Ser Lys Leu Pro Glu Pro

1250 1265 1280 1295
 ATC GTC TCC ACT GAC AGC OGC CTC TGG GTT GAA TTC OGC AGC AGC AGC AAT TGG
 Ile Val Ser Thr Asp Ser Arg Leu Trp Val Glu Phe Arg Ser Ser Ser Asn Trp

1310 1325 1340 1355
 GTT GGA AAG GGC TTC TTT GCA GTC TAC GAA GCC ATC TGC GGG GGT GAT GTG AAA
 Val Gly Lys Gly Phe Phe Ala Val Tyr Glu Ala Ile Cys Gly Gly Asp Val Lys

1370 1385 1400
 AAG GAC TAT GGC CAC ATT CAA TOG CCC AAC TAC CCA GAC GAT TAC OGG CCC AGC
 Lys Asp Tyr Gly His Ile Gln Ser Pro Asn Tyr Pro Asp Asp Tyr Arg Pro Ser

1415 1430 1445 1460
 AAA GTC TGC ATC TGG OGG ATC CAG GTG TCT GAG GGC TTC CAC GTG GGC CTC ACA
 Lys Val Cys Ile Trp Arg Ile Gln Val Ser Glu Gly Phe His Val Gly Leu Thr

1475 1490 1505
 TTC CAG TCC TTT CAG ATT GAG OGC CAC GAC AGC TGT GCC TAC GAC TAT CTG GAG
 Phe Gln Ser Phe Glu Ile Glu Arg His Asp Ser Cys Ala Tyr Asp Tyr Leu Glu

1520 1535 1550 1565
 GTG OGC GAC GGG CAC AGT GAG AGC AGC ACC CTC ATC GGG OGC TAC TGT GGC TAT
 Val Arg Asp Gly His Ser Glu Ser Ser Thr Leu Ile Gly Arg Tyr Cys Gly Tyr

1580 1595 1610 1625
 GAG AAG CCT GAT GAC ATC AAG AGC ACG TCC AGC OGC CTC TGG CTC AAG TTC GTC
 Glu Lys Pro Asp Asp Ile Lys Ser Thr Ser Ser Arg Leu Trp Leu Lys Phe Val

1640 1655 1670
 TCT GAC GGG TCC ATT AAC AAA GCG GGC TTT GCC GTC AAC TTT TTC AAA GAG GTG
 Ser Asp Gly Ser Ile Asn Lys Ala Gly Phe Ala Val Asn Phe Phe Lys Glu Val

1685 1700 1715 1730
 GAC GAG TGC TCT OGG CCC AAC OGC GGG GGC TGT GAG CAG OGC TGC CTC AAC ACC
 Asp Glu Cys Ser Arg Pro Asn Arg Gly Gly Cys Glu Gln Arg Cys Leu Asn Thr

1745 1760 1775
 CTG GGC AGC TAC AAG TGC AGC TGT GAC CCC GGG TAC GAG CTG GCC CCA GAC AAG
 Leu Gly Ser Tyr Lys Cys Ser Cys Asp Pro Gly Tyr Glu Leu Ala Pro Asp Lys

1790 1805 1820 1835
 OGC OGC TGT GAG GCT GCT TGT GGC GGA TTC CTC ACC AAG CTC AAC GGC TCC ATC
 Arg Arg Cys Glu Ala Ala Cys Gly Gly Phe Leu Thr Lys Leu Asn Gly Ser Ile

1850 1865 1880 1895
 ACC AGC OGC GGC TGG CCC AAG GAG TAC CCC CCC AAC AAG AAC TGC ATC TGG CAG
 Thr Ser Pro Gly Trp Pro Lys Glu Tyr Pro Pro Asn Lys Asn Cys Ile Trp Gln

1910 1925 1940
 CTG GTG GCC CCC ACC CAG TAC OGC ATC TCC CTG CAG TTT GAC TTC TTT GAG ACA
 Leu Val Ala Pro Thr Gln Tyr Arg Ile Ser Leu Gln Phe Asp Phe Phe Glu Thr

1955 1970 1985 2000
 GAG GGC AAT GAT GTG TGC AAG TAC GAC TTC GTG GAG GTG OGC AGT GGA CTC ACA
 Glu Gly Asn Asp Val Cys Lys Tyr Asp Phe Val Glu Val Arg Ser Gly Leu Thr

2015 2030 2045
 GCT GAC TCC AAG CTG CAT GGC AAG TTC TGT GGT TCT GAG AAG CCC GAG GTC ATC
 Ala Asp Ser Lys Leu His Gly Lys Phe Cys Gly Ser Glu Lys Pro Glu Val Ile

2060 2075 2090 2105
 ACC TCC CAG TAC AAC AAC ATG OGC GTG GAG TTC AAG TCC GAC AAC ACC GTG TCC
 Thr Ser Gln Tyr Asn Asn MET Arg Val Glu Phe Lys Ser Asp Asn Thr Val Ser

 2120 2135 2150 2165
 AAA AAG GGC TTC AAG GCC CAC TTC TTC TCA GAA AAG AGG CCA GCT CTG CAG CCC
 Lys Lys Gly Phe Lys Ala His Phe Phe Ser Glu Lys Arg Pro Ala Leu Gln Pro

 2180 2195 2210
 CCT OGG GGA OGC CCC CAC CAG CTC AAA TTC CGA GTG CAG AAA AGA AAC OGG ACC
 Pro Arg Gly Arg Pro His Gln Leu Lys Phe Arg Val Gln Lys Arg Asn Arg Thr
 (730)
 2225 2235 2245 2255 2265 2275 2285
 CCC CAG TGAGGCTGC CAGGCTOCC GGACCCCTTG TTA CTCAGGA ACCTCACTT GGACGGAATG
 Pro Gln

 2295 2305 2315 2325 2335 2345 2355
 GGATGGGGGC TTGGGTGCCC ACCAACCCCC CACCTCCACT CTGCCATTCC GGCCACCTC OCTCTGGCOG

 2365 2375 2385 2395 2405 2415 2425
 GACAGAACTG GTGCTCTCTT CTCCCCACTG TGCCCGTCCG OGGACGGGG ACCCTTCCCC GTGCCCTACC

 2435 2445 2455 2465 2475 2485 2495
 CCTCCCATTT TTGATGGTGT CTGTGACATT TCCTGTTGIG AAGTAAAAGA GGGACCCCTG CGTCCCTGCTT

CTAGA

B. hBMP-2: Class I and II

The HindIII-SacI bovine genomic bBMP-2 fragment described in Example IV B. is subcloned into an M13 vector. A ^{32}P -labeled single-stranded DNA probe is made from a template preparation of this subclone. This probe is used to screen polyadenylated RNAs from various cell and tissue sources as described above in part A. A hybridizing band corresponding to an mRNA species of approximately 3.8 kb is detected in the lane containing RNA from the human cell line U-2 OS. The HindIII-SacI fragment is labeled with ^{32}P by nick translation and used to screen the nitrocellulose filter replicas of the above-described U-2 OS cDNA library by hybridization in standard hybridization buffer at 65° overnight followed by washing in 1 X SSC, 0.1% SDS at 65° . Twelve duplicate positive clones are picked and replated for secondaries. Duplicate nitrocellulose replicas are made of the secondary plates and both sets hybridized to the bovine genomic probe as the primary screening was performed. One set of filters is then washed in 1 X SSC, 0.1% SDS; the other in 0.1 X SSC, 0.1% SDS at 65° .

Two classes of hBMP-2 cDNA clones are evident based on strong (4 recombinants) or weak (7 recombinants) hybridization signals under the more stringent washing conditions (0.1 X SSC, 0.1% SDS). All 11 recombinant bacteriophage are plaque purified, small scale DNA preparations made from plate lysates of each, and the inserts subcloned into pSP65 and into M13 for sequence analysis. Sequence analysis of the strongly hybridizing clones designated hBMP-2 Class I (also known as BMP-2) indicates that they have extensive sequence homology with the sequence given in Table III. These clones are therefore cDNA encoding the human equivalent of the protein encoded by the bBMP-2 gene whose partial sequence is given in Table III. Sequence analysis of the weakly hybridizing recombinants designated hBMP-2 Class II (also known as BMP-4) indicates that they are also quite homologous

with the sequence given in Table III at the 3' end of their coding regions, but less so in the more 5' regions. Thus they encode a human protein of similar, though not identical, structure to that above.

Full length hBMP-2 Class I cDNA clones are obtained in the following manner. The 1.5 kb insert of one of the Class II subclones (II-10-1) is isolated and radioactively labeled by nick-translation. One set of the nitrocellulose replicas of the U-2 OS cDNA library screened above (50 filters, corresponding to 1,000,000 recombinant bacteriophage) are rehybridized with this probe under stringent conditions (hybridization at 65° in standard hybridization buffer; washing at 65° in 0.2 X SSC, 0.1% SDS). All recombinants which hybridize to the bovine genomic probe which do not hybridize to the Class II probe are picked and plaque purified (10 recombinants). Plate stocks are made and small scale bacteriophage DNA preparations made. After subcloning into M13, sequence analysis indicates that 4 of these represent clones which overlap the original Class I clone. One of these, lambda U2OS-39, contains an approximately 1.5 kb insert and was deposited with the ATCC on June 16, 1987 under accession number 40345. The partial DNA sequence (compiled from lambda U2OS-39 and several other hBMP-2 Class I cDNA recombinants) and derived amino acid sequence are shown below in Table VII. Lambda U2OS-39 is expected to contain all of the nucleotide sequence necessary to encode the entire human counterpart of the protein BMP-2 Class II encoded by the bovine gene segment whose partial sequence is presented in Table III. This human cDNA hBMP-2 Class II contains an open reading frame of 1188 bp, encoding a protein of 396 amino acids. This protein of 396 amino acids has a molecular weight of 45kd based on this amino acid sequence. It is contemplated that this sequence represents the primary translation product. The protein is preceded by a 5' untranslated region of 342 bp with stop codons in all frames.

The 13 bp region preceding this 5' untranslated region represents a linker used in the cDNA cloning procedure.

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TABLE VII

| | | | | | | |
|---|------------|------------|------------|------------|------------|-------------|
| 10 | 20 | 30 | 40 | 50 | 60 | 70 |
| GTOGACTCTA | GAGTGTGTGT | CAGCACTTGG | CTGGGGACIT | CTTGAACCTG | CAGGGAGAAT | AACTTGOGCA |
| 80 | 90 | 100 | 110 | 120 | 130 | 140 |
| CCCCACTTTG | OGCOGGTGGC | TTTGCCCCAG | OGGAGCCTGC | TTGGCCATCT | COGAGCCCCA | COGCCCCCTCC |
| 150 | 160 | 170 | 180 | 190 | 200 | 210 |
| ACTCCTGGGC | CTTGCCCGAC | ACTGAGACGC | TGTTCCACGC | GTGAAAAGAG | AGACTGOGOG | GOOGGCACCC |
| 220 | 230 | 240 | 250 | 260 | 270 | 280 |
| GGGAGAAGGA | GGAGGCAAAG | AAAAGGAAOG | GACATTGGGT | CCTTGCGCCA | GGTCTTTTGA | CCAGAGTTTT |
| 290 | 300 | 310 | 320 | 330 | 340 | 350 |
| TCCATGTGGA | OGCTCTTTCA | ATGGACGTGT | CCCGOGGTGC | TTCTTAGAOG | GACTGOGGTC | TCCTAAAGGT |
| (1) | 370 | 385 | 400 | | | |
| OGACC ATG GTG GGC GGG ACC OGC TGT CTT CTA GCG TTG CTG CTT CCC CAG GTC | | | | | | |
| MET Val Ala Gly Thr Arg Cys Leu Leu Ala Leu Leu Leu Pro Gln Val | | | | | | |
| 415 | 430 | 445 | | | | |
| CTC CTG GGC GGC GCG GCT GGC CTC GTT CCG GAG CTG GGC OGC AGG AAG TTC GCG | | | | | | |
| Leu Leu Gly Gly Ala Ala Gly Leu Val Pro Glu Leu Gly Arg Arg Lys Phe Ala | | | | | | |
| 460 | 475 | 490 | 505 | | | |
| GCG GCG TOG TOG GGC OGC CCC TCA TCC CAG CCC TCT GAC GAG GTC CTG AGC GAG | | | | | | |
| Ala Ala Ser Ser Gly Arg Pro Ser Ser Gln Pro Ser Asp Glu Val Leu Ser Glu | | | | | | |
| 520 | 535 | 550 | 565 | | | |
| TTC GAG TTG OGG CTG CTC AGC ATG TTC GGC CTG AAA CAG AGA CCC ACC CCC AGC | | | | | | |
| Phe Glu Leu Arg Leu Leu Ser MET Phe Gly Leu Lys Gln Arg Pro Thr Pro Ser | | | | | | |
| 580 | 595 | 610 | | | | |
| AGG GAC GGC GTG GTG CCC CCC TAC ATG CTA GAC CTG TAT OGC AGG CAC TCG GGT | | | | | | |
| Arg Asp Ala Val Val Pro Pro Tyr MET Leu Asp Leu Tyr Arg Arg His Ser Gly | | | | | | |
| 625 | 640 | 655 | 670 | | | |
| CAG CCG GGC TCA CCC GGC CCA GAC CAC OGG TTG GAG AGG GCA GCC AGC CGA GCC | | | | | | |
| Gln Pro Gly Ser Pro Ala Pro Asp His Arg Leu Glu Arg Ala Ala Ser Arg Ala | | | | | | |
| 685 | 700 | 715 | | | | |
| AAC ACT GTG OGC AGC TTC CAC CAT GAA GAA TCT TTG GAA GAA CTA CCA GAA ACG | | | | | | |
| Asn Thr Val Arg Ser Phe His His Glu Glu Ser Leu Glu Glu Leu Pro Glu Thr | | | | | | |

730 745 760 775
 AGT GGG AAA ACA ACC CGG AGA TTC TTC TTT AAT TTA AGT TCT ATC CCC ACG GAG
 Ser Gly Lys Thr Thr Arg Arg Phe Phe Phe Asn Leu Ser Ser Ile Pro Thr Glu
 790 805 820 835
 GAG TTT ATC ACC TCA GCA GAG CTT CAG GTT TTC CGA GAA CAG ATG CAA GAT GCT
 Glu Phe Ile Thr Ser Ala Glu Leu Gln Val Phe Arg Glu Gln MET Gln Asp Ala
 850 865 880
 TTA GGA AAC AAT AGC AGT TTC CAT CAC CGA ATT AAT ATT TAT GAA ATC ATA AAA
 Leu Gly Asn Asn Ser Ser Phe His His Arg Ile Asn Ile Tyr Glu Ile Ile Lys
 895 910 925 940
 CCT GCA ACA GCC AAC TCG AAA TTC CCC GTG ACC AGT CTT TTG GAC ACC AGG TTG
 Pro Ala Thr Ala Asn Ser Lys Phe Pro Val Thr Ser Leu Leu Asp Thr Arg Leu
 955 970 985
 GTG AAT CAG AAT GCA AGC AGG TGG GAA AGT TTT GAT GTC ACC CCC GCT GTG ATG
 Val Asn Gln Asn Ala Ser Arg Trp Glu Ser Phe Asp Val Thr Pro Ala Val MET
 1000 1015 1030 1045
 CGG TGG ACT GCA CAG GCA CAC GCC AAC CAT GGA TTC GTG GTG GAA GTG GCC CAC
 Arg Trp Thr Ala Gln Gly His Ala Asn His Gly Phe Val Val Glu Val Ala His
 1060 1075 1090 1105
 TTG GAG GAG AAA CAA GGT GTC TCC AAG AGA CAT GTT AGG ATA AGC AGG TCT TTG
 Leu Glu Glu Lys Gln Gly Val Ser Lys Arg His Val Arg Ile Ser Arg Ser Leu
 1120 1135 1150
 CAC CAA GAT GAA CAC AGC TGG TCA CAG ATA AGG CCA TTG CTA GTA ACT TTT GGC
 His Gln Asp Glu His Ser Trp Ser Gln Ile Arg Pro Leu Leu Val Thr Phe Gly
 1165 1180 1195 1210
 CAT GAT GGA AAA GGG CAT CCT CTC CAC AAA AGA GAA AAA CGT CAA GCC AAA CAC
 His Asp Gly Lys Gly His Pro Leu His Lys Arg Glu Lys Arg Gln Ala Lys His
 1225 1240 1255
 AAA CAG CGG AAA CGC CTT AAG TCC AGC TGT AAG AGA CAC CCT TTG TAC GTG GAC
 Lys Gln Arg Lys Arg Leu Lys Ser Ser Cys Lys Arg His Pro Leu Tyr Val Asp
 1270 1285 1300 1315
 TTC AGT GAC GTG GGG TGG AAT GAC TGG ATT GTG GCT CCC CCG GGG TAT CAC GCC
 Phe Ser Asp Val Gly Trp Asn Asp Trp Ile Val Ala Pro Pro Gly Tyr His Ala
 1330 1345 1360 1375
 TTT TAC TGC CAC GGA GAA TGC CCT TTT CCT CTG GCT GAT CAT CTG AAC TCC ACT
 Phe Tyr Cys His Gly Glu Cys Pro Phe Pro Leu Ala Asp His Leu Asn Ser Thr
 1390 1405 1420
 AAT CAT GCC ATT GTT CAG ACG TTG GTC AAC TCT GTT AAC TCT AAG ATT CCT AAG
 Asn His Ala Ile Val Gln Thr Leu Val Asn Ser Val Asn Ser Lys Ile Pro Lys

1435 1450 1465 1480
GCA TGC TGT GTC CCG ACA GAA CTC AGT GCT ATC TOG ATG CTG TAC CTT GAC GAG
Ala Cys Cys Val Pro Thr Glu Leu Ser Ala Ile Ser MET Leu Tyr Leu Asp Glu

1495 1510 1525
AAT GAA AAG GTT GTA TTA AAG AAC TAT CAG GAC ATG GTT GTG GAG GGT TGT GGG
Asn Glu Lys Val Val Leu Lys Asn Tyr Gln Asp MET Val Val Glu Gly Cys Gly

1540(396) 1553 1563 1573 1583 1593 1603
TGT CGC TAGTACAGCA AAATTAAATA CATAAATATA TATATATATA TATATTTTAG AAAAAAGAAA
Cys=Arg

AAAA:

Full-length hBMP-2 Class II human cDNA clones are obtained in the following manner. The 200 bp EcoRI-SacI fragment from the 5' end of the Class II recombinant II-10-1 is isolated from its plasmid subclone, labeled by nick-translation, and hybridized to a set of duplicate nitrocellulose replicas of the U-2 OS cDNA library (25 filters/set; representing 500,000 recombinants). Hybridization and washing are performed under stringent conditions as described above. 16 duplicate positives are picked and replated for secondaries. Nitrocellulose filter replicas of the secondary plates are made and hybridized to an oligonucleotide which was synthesized to correspond to the sequence of II-10-1 and is of the following sequence:

CGGGCGCTCAGGATACTCAAGACCAGTGCTG

Hybridization is in standard hybridization buffer AT 50° C with washing at 50° in 1 X SSC, 0.1% SDS. 14 recombinant bacteriophage which hybridize to this oligonucleotide are plaque purified. Plate stocks are made and small scale bacteriophage DNA preparations made. After sucloning 3 of these into M13, sequence analysis indicates that they represent clones which overlap the original Class II clone. One of these, lambda U2OS-3, was deposited with the ATCC under accession number 40342 on June 16, 1987. U2OS-3 contains an insert of approximately 1.8 kb. The partial DNA sequence and derived amino acid sequence of U2OS-3 are shown below in Table VIII. This clone is expected to contain all of the nucleotide sequence necessary to encode the entire human BMP-2 Class II protein. This cDNA contains an open reading frame of 1224 bp, encoding a protein of 408 amino acids, preceded by a 5' untranslated region of 394 bp with stop codons in all frames, and contains a 3' untranslated region of 308 bp following the in-frame stop codon. The 8 bp region preceding the 5' untranslated region represents a linker used in the cDNA cloning procedure. This protein of 408 amino acids has molecular weight of 47kd and is contemplated to represent the

primary translation product.

TABLE VIII

| | | | | | | |
|---|------------|------------|------------|------------|----------------|-------------|
| 10 | 20 | 30 | 40 | 50 | 60 | 70 |
| CTCTAGAGGG | CAGAGGAGGA | GGGAGGGAGG | GAAGGAGOGC | GGAGCCOGGC | COGGAAGCTA | GGTGAGTGTG |
| 80 | 90 | 100 | 110 | 120 | 130 | 140 |
| GCATCOGAGC | TGAGGGAOGC | GAGCCTGAGA | CGCOGCTGCT | GCTCOGGCTG | AGTATCTAGC | TTGTCTCCCC |
| 150 | 160 | 170 | 180 | 190 | 200 | 210 |
| GATGGGATTG | COGTCCAAGC | TATCTOGAGC | CTGCAGOGCC | ACAGTCCCOG | GCCCTOGCCC | AGGTTCACTG |
| 220 | 230 | 240 | 250 | 260 | 270 | 280 |
| CAACOGTTCA | GAGGTCCCCA | GGAGCTGCTG | CTGGOGAGCC | CGCTACTGCA | GGGACCTATG | GAGCCATTCC |
| 290 | 300 | 310 | 320 | 330 | 340 | 350 |
| GTAGTGCCAT | COOGAGCAAC | GCACTGCTGC | AGCTTCOCTG | AGCCTTTCCA | GCAAGTTTGT | TCAAGATTGG |
| 360 | 370 | 380 | 390 | 400 | (1) | |
| CTGTCAAGAA | TCATGGACTG | TTATTATATG | CCTTGTITTC | TGTCAAGACA | CC ATG ATT CCT | MET Ile Pro |
| 417 | 432 | 447 | 462 | | | |
| GGT AAC CGA ATG CTG ATG GTC GTT TTA TTA TGC CAA GTC CTG CTA GGA GGC GCG | | | | | | |
| Gly Asn Arg MET Leu MET Val Val Leu Leu Cys Gln Val Leu Leu Gly Gly Ala | | | | | | |
| 477 | 492 | 507 | | | | |
| AGC CAT GCT AGT TTG ATA CCT GAG ACG GGG AAG AAA AAA GTC GCC GAG ATT CAG | | | | | | |
| Ser His Ala Ser Leu Ile Pro Glu Thr Gly Lys Lys Lys Val Ala Glu Ile Gln | | | | | | |
| 522 | 537 | 552 | 567 | | | |
| GGC CAC GCG GGA GGA OGC OGC TCA GGG CAG AGC CAT GAG CTC CTG OGG GAC TTC | | | | | | |
| Gly His Ala Gly Gly Arg Arg Ser Gly Gln Ser His Glu Leu Leu Arg Asp Phe | | | | | | |
| 582 | 597 | 612 | 627 | | | |
| GAG GCG ACA CTT CTG CAG ATG TTT GGG CTG OGC OGC OGC COG CAG CCT AGC AAG | | | | | | |
| Glu Ala Thr Leu Leu Gln MET Phe Gly Leu Arg Arg Arg Pro Gln Pro Ser Lys | | | | | | |
| 642 | 657 | 672 | | | | |
| AGT GCC GTC ATT COG GAC TAC ATG OGG GAT CTT TAC OGG CTT CAG TCT GGG GAG | | | | | | |
| Ser Ala Val Ile Pro Asp Tyr MET Arg Asp Leu Tyr Arg Leu Gln Ser Gly Glu | | | | | | |
| 687 | 702 | 717 | 732 | | | |
| GAG GAG GAA GAG CAG ATC CAC AGC ACT GGT CTT GAG TAT CCT GAG OGC COG GCC | | | | | | |
| Glu Glu Glu Glu Gln Ile His Ser Thr Gly Leu Glu Tyr Pro Glu Arg Pro Ala | | | | | | |

47

747 762 777
 AGC OGG GCC AAC ACC GTG AGG AGC TTC CAC CAC GAA GAA CAT CTG GAG AAC ATC
 Ser Arg Ala Asn Thr Val Arg Ser Phe His His Glu Glu His Leu Glu Asn Ile

792 807 822 837
 CCA GGG ACC AGT GAA AAC TCT GCT TTT OGT TTC CTC TTT AAC CTC AGC AGC ATC
 Pro Gly Thr Ser Glu Asn Ser Ala Phe Arg Phe Leu Phe Asn Leu Ser Ser Ile

852 867 882 897
 CCT GAG AAC GAG GTG ATC TCC TCT GCA GAG CTT OGG CTC TTC OGG GAG CAG GTG
 Pro Glu Asn Glu Val Ile Ser Ser Ala Glu Leu Arg Leu Phe Arg Glu Gln Val

912 927 942
 GAC CAG GGC OCT GAT TGG GAA AGG GGC TTC CAC OGT ATA AAC ATT TAT GAG GTT
 Asp Gln Gly Pro Asp Trp Glu Arg Gly Phe His Arg Ile Asn Ile Tyr Glu Val

957 972 987 1002
 ATG AAG CCC CCA GCA GAA GTG GTG OCT GGG CAC CTC ATC ACA OGA CTA CTG GAC
 MET Lys Pro Pro Ala Glu Val Val Pro Gly His Leu Ile Thr Arg Leu Leu Asp

1017 1032 1047
 ACG AGA CTG GTC CAC CAC AAT GTG ACA OGG TGG GAA ACT TTT GAT GTG AGC CCT
 Thr Arg Leu Val His His Asn Val Thr Arg Trp Glu Thr Phe Asp Val Ser Pro

1062 1077 1092 1107
 GCG GTC CTT OGC TGG ACC OGG GAG AAG CAG CCA AAC TAT GGG CTA GCC ATT GAG
 Ala Val Leu Arg Trp Thr Arg Glu Lys Gln Pro Asn Tyr Gly Leu Ala Ile Glu

1122 1137 1152 1167
 GTG ACT CAC CTC CAT CAG ACT OGG ACC CAC CAG GGC CAG CAT GTC AGG ATT AGC
 Val Thr His Leu His Gln Thr Arg Thr His Gln Gly Gln His Val Arg Ile Ser

1182 1197 1212
 OGA TOG TTA OCT CAA GGG AGT GGG AAT TGG GCC CAG CTC OGG CCC CTC CTG GTC
 Arg Ser Leu Pro Gln Gly Ser Gly Asn Trp Ala Gln Leu Arg Pro Leu Leu Val

1227 1242 1257 1272
 ACC TTT GGC CAT GAT GGC OGG GGC CAT GCC TTG AOC OGA OGC OGG AGG GCC AAG
 Thr Phe Gly His Asp Gly Arg Gly His Ala Leu Thr Arg Arg Arg Ala Lys

1287 1302 1317
 OGT AGC OCT AAG CAT CAC TCA CAG OGG GCC AGG AAG AAG AAT AAG AAC TGC OGG
 Arg Ser Pro Lys His His Ser Gln Arg Ala Arg Lys Lys Asn Lys Asn Cys Arg

1332 1347 1362 1377
 OGC CAC TOG CTC TAT GTG GAC TTC AGC GAT GTG GGC TGG AAT GAC TGG ATT GTG
 Arg His Ser Leu Tyr Val Asp Phe Ser Asp Val Gly Trp Asn Asp Trp Ile Val

1392 1407 1422 1437
 GCC CCA CCA GGC TAC CAG GCC TTC TAC TGC CAT GGG GAC TGC CCC TTT CCA CTG
 Ala Pro Pro Gly Tyr Gln Ala Phe Tyr Cys His Gly Asp Cys Pro Phe Pro Leu

48

1452 1467 1482
 GCT GAC CAC CTC AAC TCA ACC AAC CAT GCC ATT GTG CAG ACC CTG GTC AAT TCT
 Ala Asp His Leu Asn Ser Thr Asn His Ala Ile Val Gln Thr Leu Val Asn Ser

1497 1512 1527 1542
 GTC AAT TCC AGT ATC CCC AAA GCC TGT TGT GTG CCC ACT GAA CTG AGT GCC ATC
 Val Asn Ser Ser Ile Pro Lys Ala Cys Cys Val Pro Thr Glu Leu Ser Ala Ile

1557 1572 1587
 TCC ATG CTG TAC CTG GAT GAG TAT GAT AAG GTG GTA CTG AAA AAT TAT CAG GAG
 Ser MET Leu Tyr Leu Asp Glu Tyr Asp Lys Val Val Leu Lys Asn Tyr Gln Glu

1602 1617 (408) 1636 1646 1656
 ATG GTA GTA GAG GGA TGT GGG TGC CGC TGAGATCAGG CAGTCCCTGA GCATAGACAG
 MET Val Val Glu Gly Cys Gly Cys Arg

1666 1676 1686 1696 1706 1716 1726
 ATATACACAC CACACACACA CACCACATAC ACCACACACA CACGTTCCCA TCCACTCACC CACACACTAC

1736 1746 1756 1766 1776 1786 1796
 ACAGACTGCT TCCTTATAGC TGGACTTTTA TTTAAAAAAA AAAAAAAA AATGCAGAAA ATCCCTAAAC

1806 1816 1826 1836 1846 1856 1866
 ATTCACCTTG ACCTTATTTA TGACTTTACG TGCAAATGTT TTGACCATAT TGATCATATA TTTTGACAAA

1876 1886 1896 1906 1916 1926 1936
 ATATATTTAT AACTACGTAT TAAAAGAAAA AAATAAAATG AGTCATTATT TTAAAAAAA AAAAAAACT

1946
 CTAGAGTGA CGGAATTC

The sequences of BMP-2 Class I and II, as well as BMP-3 as shown in Tables III, IV, VII and VIII have significant homology to the beta (B) and beta (A) subunits of the inhibins. The inhibins are a family of hormones which are presently being investigated for use in contraception. See, A. J. Mason et al, Nature, 318:659-663 (1985). To a lesser extent they are also homologous to Mullerian inhibiting substance (MIS), a testicular glycoprotein that causes regression of the Mullerian duct during development of the male embryo and transforming growth factor-beta (TGF-b) which can inhibit or stimulate growth of cells or cause them to differentiate. Furthermore, the sequence of Table VII encoding hBMP-2 Class II has significant homology to the Drosophila decapentaplegic (DPP-C) locus transcript. See, J. Massague, Cell, 49:437-438 (1987); R. W. Padgett et al, Nature, 325:81-84 (1987); R. L. Cate et al, Cell 45: 685-698 (1986). It is considered possible therefore that BMP-2 Class II is the human homolog of the protein made from this transcript from this developmental mutant locus.

C. BMP-3

Because bovine and human bone growth factor genes are presumed to be significantly homologous, oligonucleotide probes which have been shown to hybridize to the bovine DNA sequence of Table IV.A and IV.B are used to screen a human genomic library. A human genomic library (Toole et al., supra) is screened using these probes, and presumptive positives are isolated and DNA sequence obtained as described above. Evidence that this recombinant encodes a portion of the human bone inductive factor molecule relies on the bovine/human protein and gene structure homologies.

Once a recombinant bacteriophage containing DNA encoding a portion of the human BMP-3 molecule is obtained the human coding sequence is used as a probe as described in Example V (A) to identify a human cell line or tissue which synthesizes BMP-3. mRNA is selected by oligo (dT) cellulose

chromatography and cDNA is synthesized and cloned in lambda gt10 by established techniques (Toole et al., supra).

Alternatively, the entire gene encoding this human bone inductive factor can be identified and obtained in additional recombinant clones if necessary. Additional recombinants containing further 3' or 5' regions of this human bone inductive factor gene can be obtained by identifying unique DNA sequences at the end(s) of the original clone and using these as probes to rescreen the human genomic library. The gene can then be reassembled in a single plasmid by standard molecular biology techniques and amplified in bacteria. The entire human BMP-3 factor gene can then be transferred to an appropriate expression vector. The expression vector containing the gene is then transfected into a mammalian cell, e.g. monkey COS cells, where the human gene is transcribed and the RNA correctly spliced. Media from the transfected cells are assayed for bone inductive factor activity as described herein as an indication that the gene is complete. mRNA is obtained from these cells and cDNA synthesized from this mRNA source and cloned. The procedures described above may similarly be employed to isolate other species' bone inductive factor of interest by utilizing the bovine bone inductive factor and/or human bone inductive factor as a probe source. Such other species' bone inductive factor may find similar utility in, inter alia, fracture repair.

EXAMPLE VI

Expression of Bone Inductive Factors.

In order to produce bovine, human or other mammalian bone inductive factors, the DNA encoding it is transferred into an appropriate expression vector and introduced into mammalian cells by conventional genetic engineering techniques.

One skilled in the art can construct mammalian expression vectors by employing the sequence of Tables II-

VIII or other modified sequences and known vectors, such as pCD [Okayama et al., Mol. Cell Biol., 2:161-170 (1982)] and pJL3; pJL4 [Gough et al., EMBO J., 4:645-653 (1985)]. The transformation of these vectors into appropriate host cells can result in expression of osteoinductive factors. One skilled in the art could manipulate the sequences of Tables II-VIII by eliminating or replacing the mammalian regulatory sequences flanking the coding sequence with bacterial sequences to create bacterial vectors for intracellular or extracellular expression by bacterial cells. For example, the coding sequences could be further manipulated (e.g. ligated to other known linkers or modified by deleting non-coding sequences there-from or altering nucleotides therein by other known techniques). The modified bone inductive factor coding sequence could then be inserted into a known bacterial vector using procedures such as described in T. Taniguchi et al., Proc. Natl Acad. Sci. USA, 77:5230-5233 (1980). This exemplary bacterial vector could then be transformed into bacterial host cells and bone inductive factor expressed thereby. For a strategy for producing extracellular expression of bone inductive factor in bacterial cells., see, e.g. European patent application EPA 177,343.

Similar manipulations can be performed for the construction of an insect vector [See, e.g. procedures described in published European patent application 155,476] for expression in insect cells. A yeast vector could also be constructed employing yeast regulatory sequences for intracellular or extracellular expression of the factors of the present invention by yeast cells. [See, e.g., procedures described in published PCT application WO86/00639 and European patent application EPA 123,289].

A method for producing high levels of an osteoinductive factor of the invention from mammalian cells involves the construction of cells containing multiple copies of the heterologous bone inductive factor gene. The heterologous gene

can be linked to an amplifiable marker, e.g. the dihydrofolate reductase (DHFR) gene for which cells containing increased gene copies can be selected for propagation in increasing concentrations of methotrexate (MTX) according to the procedures of Kaufman and Sharp, J. Mol. Biol., 159:601-629 (1982). This approach can be employed with a number of different cell types.

For example, a plasmid containing a DNA sequence for a bone inductive factor of the invention in operative association with other plasmid sequences enabling expression thereof and the DHFR expression plasmid pAdA26SV(A)3 [Kaufman and Sharp, Mol. Cell. Biol., 2:1304 (1982)] can be co-introduced into DHFR-deficient CHO cells, DUKX-BII, by calcium phosphate coprecipitation and transfection. DHFR expressing transformants are selected for growth in alpha media with dialyzed fetal calf serum, and subsequently selected for amplification by growth in increasing concentrations of MTX (sequential steps in 0.02, 0.2, 1.0 and 5uM MTX) as described in Kaufman et al., Mol Cell Biol., 5:1750 (1983). Transformants are cloned, and biologically active bone inductive factor expression is monitored by rat bone formation assay. Bone inductive factor expression should increase with increasing levels of MTX resistance. Similar procedures can be followed to produce other bone inductive factors.

Alternatively, the human gene is expressed directly, as described above. Active bone inductive factor may be produced in bacteria or yeast cells. However the presently preferred expression system for biologically active recombinant human bone inductive factor is stably transformed CHO cells.

As one specific example, to produce the human bone inductive factor (hBMP-1) of Example V, the insert of U2OS-1 is released from the vector arms by digestion with Sal I and subcloned into the mammalian expression vector pMT2CX digested with Xho I. Plasmid DNA from this subclone is transfected into COS cells by the DEAE-dextran procedure [Sompayrac and

Danna PNAS 78:7575-7578 (1981); Luthman and Magnusson, Nucl.Acids Res. 11: 1295-1308 (1983)]. Serum-free 24 hr. conditioned medium is collected from the cells starting 40-70 hr. post-transfection.

The mammalian expression vector pMT2 Cla-Xho (pMT2 CX) is a derivative of p91023 (b) (Wong et al., Science 228:810-815, 1985) differing from the latter in that it contains the ampicillin resistance gene in place of the tetracycline resistance gene and further contains a XhoI site for insertion of cDNA clones. The functional elements of pMT2 Cla-Xho have been described (Kaufman, R.J., 1985, Proc. Natl. Acad. Sci. USA 82:689-693) and include the adenovirus VA genes, the SV40 origin of replication including the 72 bp enhancer, the adenovirus major late promoter including a 5' splice site and the majority of the adenovirus tripartite leader sequence present on adenovirus late mRNAs, a 3' splice acceptor site, a DHFR insert, the SV40 early polyadenylation site (SV40), and pBR322 sequences needed for propagation in E. coli.

Plasmid pMT2 Cla-Xho is obtained by EcoRI digestion of pMT2-VWF, which has been deposited with the American Type Culture Collection (ATCC), Rockville, MD (USA) under accession number ATCC 67122. EcoRI digestion excises the cDNA insert present in pMT2-VWF, yielding pMT2 in linear form which can be ligated and used to transform E. coli HB 101 or DH-5 to ampicillin resistance. Plasmid pMT2 DNA can be prepared by conventional methods. pMT2CX is then constructed by digesting pMT2 with Eco RV and XbaI, treating the digested DNA with Klenow fragment of DNA polymerase I, and ligating Cla linkers (NEBiolabs, CATCGATG). This removes bases 2266 to 2421 starting from the Hind III site near the SV40 origin of replication and enhancer sequences of pMT2. Plasmid DNA is then digested with EcoRI, blunted as above, and ligated to an EcoRI adapter,

5' PO₄-AATTCCTCGAGAGCT 3'

3' GGAGCTCTCGA 5'

digested with XhoI, and ligated, yielding pMT2 Cla-Xho, which may then be used to transform E. coli to ampicillin resistance. Plasmid pMT2 Cla-Xho DNA may be prepared by conventional methods.

Example VII

Biological Activity of Expressed Bone Inductive Factor

A. BMP-1

To measure the biological activity of the expressed bone inductive factor (hBMP-1) obtained in Example VI above. The factor is partially purified on a Heparin Sepharose column. 4 ml of transfection supernatant from one 100 mm dish is concentrated approximately 10 fold by ultrafiltration on a YM 10 membrane and then dialyzed against 20mM Tris, 0.15 M NaCl, pH 7.4 (starting buffer). This material is then applied to a 1.1 ml Heparin Sepharose column in starting buffer. Unbound proteins are removed by an 8 ml wash of starting buffer, and bound proteins, including BMP-1, are desorbed by a 3-4 ml wash of 20 mM Tris, 2.0 M NaCl, pH 7.4.

The proteins bound by the Heparin column are concentrated approximately 10-fold on a Centricon 10 and the salt reduced by diafiltration with 0.1% trifluoroacetic acid. The appropriate amount of this solution is mixed with 20 mg of rat matrix and then assayed for in vivo bone and cartilage formation as previously described in Example III. A mock transfection supernatant fractionation is used as a control.

The implants containing rat matrix to which specific amounts of human BMP-1 have been added are removed from rats after seven days and processed for histological evaluation. Representative sections from each implant are stained for the presence of new bone mineral with von Kossa and acid fuschin, and for the presence of cartilage-specific matrix formation using toluidine blue. The types of cells present within the section, as well as the extent to which these cells display phenotype are evaluated.

Addition of human BMP-1 to the matrix material resulted in formation of cartilage-like nodules at 7 days post implantation. The chondroblast-type cells were recognizable by shape and expression of metachromatic matrix. The amount of activity observed for human BMP-1 was dependent upon the amount of human BMP-1 protein added to the matrix. Table IX illustrates the dose-response relationship of human BMP-1 protein to the amount of bone induction observed.

Table IX

| <u>IMPLANT NUMBER</u> | <u>AMOUNT USED</u> (equivalent of ml transfection media) | <u>HISTOLOGICAL SCORE</u> |
|-----------------------|--|---------------------------|
| 876-134-1 | 10 BMP-1 | C+2 |
| 876-134-2 | 3 BMP-1 | C+1 |
| 876-134-3 | 1 BMP-1 | C +/- |
| 876-134-4 | 10 MOCK | C - |
| 876-134-5 | 3 MOCK | C - |
| 876-134-6 | 1 MOCK | C - |

Cartilage (c) activity was scored on a scale from 0(-) to 5.

Similar levels of activity are seen in the Heparin Sepharose fractionated COS cell extracts. Partial purification is accomplished in a similar manner as described above, except that 6 M urea is included in all the buffers. Further, in a rat bone formation assay as described above, BMP-2 has similarly demonstrated chondrogenic activity.

The procedures described above may be employed to isolate other bone inductive factors of interest by utilizing the bovine bone inductive factors and/or human bone inductive factors as a probe source. Such other bone inductive factors may find similar utility in, inter alia, fracture repair.

The foregoing descriptions detail presently preferred embodiments of the present invention. Numerous modifications

and variations in practice thereof are expected to occur to those skilled in the art upon consideration of these descriptions. Those modifications and variations are believed to be encompassed within the claims appended hereto.

International Application No: PCT/

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MICROORGANISMS

Optional: Sheet in connection with the microorganism referred to on page _____, line _____ of the description *

A. IDENTIFICATION OF DEPOSIT *Further deposits are identified on an additional sheet ☐ *

Name of depositary institution *

American Type Culture Collection

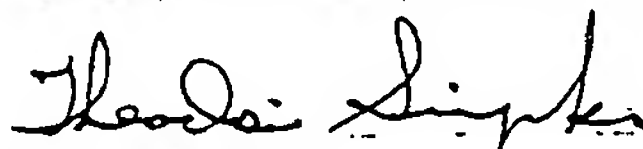
Address of depositary institution (including postal code and country) *

12301 Parklawn Drive
Rockville, Maryland 20852 USA

| <u>Name of Deposit</u> | <u>ATCC No.</u> | <u>Referred to on page/line</u> | <u>Date of Deposit</u> |
|------------------------|-----------------|---------------------------------|------------------------|
| LP-H1 | 40311 | 29/20 | March 4, 1987 |
| bP50 | 40295 | 20/3 | December 15, 1986 |
| bP-21 | 40310 | 22/18 | March 4, 1987 |
| U2OS-3 | 40342 | 44/22 | June 16, 1987 |
| Lambda U2-OS-1 | 40343 | 32/33 | June 16, 1987 |
| Lambda BP819 | 40344 | 25/23 | June 16, 1987 |
| U2OS-39 | 40345 | 39/21 | June 16, 1987 |

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE * (if the indications are not for all designated States)**D. SEPARATE FURNISHING OF INDICATIONS *** (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)
(Authorized Officer)☐ The date of receipt (from the applicant) by the International Bureau ¹⁰

(Authorized Officer)

WHAT IS CLAIMED IS:

1.. A pharmaceutical composition comprising a protein selected from the group consisting of:

- (a) BMP-1;
- (b) BMP-2 Class I;
- (c) BMP-2 Class II;
- (d) BMP-3; and

mixtures thereof, in a pharmaceutically acceptable vehicle.

2.. A composition of Claim 1 wherein said protein is BMP-1.

3.. A composition of Claim 1 wherein said protein is BMP-2 Class I.

4. A composition of Claim 1 wherein said protein is BMP-2 Class II.

5. A composition of Claim 1 wherein said protein is BMP-3.

6. The pharmaceutical composition of Claim 1 further comprising a matrix capable of delivering the composition to the site of the bone defect and providing a structure for inducing bone formation.

7.. The composition of Claim 6 wherein said matrix comprises a material selected from the group consisting of hydroxyapatite, collagen, polylactic acid and tricalcium phosphate.

8.. A method for inducing bone formation in a patient in need of same comprising administering to said patient an effective amount of a composition of Claim 1-7.

9. A process for producing BMP-1 comprising culturing in a suitable culture medium a cell line transformed with a DNA sequence encoding BMP-1, said DNA sequence being in relative association with an expression control sequence therefor, and isolating BMP-1 from said culture medium.

10. A process according to Claim 9 wherein said DNA sequence comprises substantially the nucleotide sequence of Table VI.

11. A process for producing BMP-2 Class I comprising culturing in a suitable culture medium a cell line transformed

with a DNA sequence encoding BMP-2 Class I, said DNA sequence being in relative association with an expression control sequence therefor, and isolating BMP-2 Class I from said culture medium.

12. A process for according to Claim 11 wherein said DNA sequence comprises substantially the nucleotide sequence of Table VII.

13. A process for producing BMP-2 Class II comprising culturing in a suitable culture medium a cell line transformed with a DNA sequence encoding BMP-2 Class II, said DNA sequence being in relative association with an expression control sequence therefor, and isolating BMP-2 Class II from said culture medium.

14. A process according to Claim 13 wherein said DNA sequence comprises substantially the nucleotide sequence of Table VIII.

15. A process for producing BMP-3 comprising culturing in a suitable culture medium a cell line transformed with a DNA sequence encoding BMP-3, said DNA sequence being in relative association with an expression control sequence therefor and isolating BMP-3 from said culture medium.

16. A process according to Claim 15 wherein said DNA sequence comprises substantially the nucleotide sequence of Table IVA and IVB.

17. A cDNA sequence encoding BMP-1 comprising substantially the nucleotide sequence of Table VI or a sequence which hybridize thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-1.

18. A cDNA sequence encoding BMP-2 Class I comprising substantially the nucleotide sequence of Table VII or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-2 Class I.

19. A cDNA sequence encoding BMP-2 Class II comprising

substantially the nucleotide sequence of Table VIII or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-2 Class II.

20. A cDNA sequence encoding BMP-3 comprising substantially the nucleotide sequence of Table IVA and IVB or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-3.

AMENDED CLAIMS

[received by the International Bureau on 8 December 1987 (08.12.87)
original claims 6, 8, 10, 12, 14, 16-20 amended;
new claims 21-23 added; other claims unchanged (13 pages)]

1. A pharmaceutical composition comprising a protein selected from the group consisting of:

- (a) BMP-1;
- (b) BMP-2 Class I;
- (c) BMP-2 Class II;
- (d) BMP-3; and

mixtures thereof, in a pharmaceutically acceptable vehicle.

2.. A composition of Claim 1 wherein said protein is BMP-1..

3.. A composition of Claim 1 wherein said protein is BMP-2 Class I.

4. A composition of Claim 1 wherein said protein is BMP-2 Class II.

5. A composition of Claim 1 wherein said protein is BMP-3.

6. The pharmaceutical composition of Claim 1 further comprising a matrix capable of delivering the composition to the site of the bone or cartilage defect and providing a structure for inducing bone or cartilage formation.

7. The composition of Claim 6 wherein said matrix comprises a material selected from the group consisting of hydroxyapatite, collagen, polylactic acid and tricalcium phosphate.

8. A method for inducing bone or cartilage formation in a patient in need of same comprising administering to said patient an effective amount of a composition of Claim 1-7.

9. A process for producing BMP-1 comprising the steps of culturing in a suitable culture medium a host cell transformed with a DNA sequence encoding BMP-1, said DNA sequence being in relative association with an expression control sequence therefor; and isolating said BMP-1 therefrom.

10. A process according to Claim 9 wherein said DNA sequence comprises substantially the nucleotide sequence as follows:

| | | | |
|---|-----------|-----------|---------------------------------------|
| 10 | 20 | 30 | 50 |
| CTAGAGGCG | CTTCCCTCG | CGCGCCCCG | CCAGC ATG CCC GGC GTG GCC CGC CTG CCG |
| | | | MET Pro Gly Val Ala Arg Leu Pro |
| 65 | 80 | 95 | 110 |
| CTG CTG CTC GGG CTG CTG CTG CTC CCG CGT CCC GGC CGG CCG CTG GAC TTG GCC | | | |
| Leu Leu Leu Gly Leu Leu Leu Leu Pro Arg Pro Gly Arg Pro Leu Asp Leu Ala | | | |
| 125 | 140 | 155 | |
| GAC TAC ACC TAT GAC CTG GCG GAG GAG GAC GAC TCG GAG CCC CTC AAC TAC AAA | | | |
| Asp Tyr Thr Tyr Asp Leu Ala Glu Glu Asp Asp Ser Glu Pro Leu Asn Tyr Lys | | | |
| 170 | 185 | 200 | 215 |
| GAC CCC TGC AAG GCG GCT GCC TTT CTT GGG GAC ATT GCC CTG GAC GAA GAG GAC | | | |
| Asp Pro Cys Lys Ala Ala Ala Phe Leu Gly Asp Ile Ala Leu Asp Glu Glu Asp | | | |
| 230 | 245 | 260 | 275 |
| CTG AGG GCC TTC CAG GTA CAG CAG GCT GTG GAT CTC AGA CGG CAC ACA GCT CGT | | | |
| Leu Arg Ala Phe Gln Val Gln Gln Ala Val Asp Leu Arg Arg His Thr Ala Arg | | | |
| 290 | 305 | 320 | |
| AAG TCC TCC ATC AAA GCT GCA GTT CCA GGA AAC ACT TCT ACC CCC AGC TGC CAG | | | |
| Lys Ser Ser Ile Lys Ala Ala Val Pro Gly Asn Thr Ser Thr Pro Ser Cys Gln | | | |
| 335 | 350 | 365 | 380 |
| AGC ACC AAC GGG CAG CCT CAG AGG GGA GCC TGT GCG AGA TCG AGA GGT AGA TCC | | | |
| Ser Thr Asn Gly Gln Pro Gln Arg Gly Ala Cys Gly Arg Trp Arg Gly Arg Ser | | | |
| 395 | 410 | 425 | |
| CGT AGC CGG CGG GCG GCG ACG TCC CGA CCA GAG CGT GTG TGG CCC GAT GCG GTC | | | |
| Arg Ser Arg Arg Ala Ala Thr Ser Arg Pro Glu Arg Val Trp Pro Asp Gly Val | | | |
| 440 | 455 | 470 | 485 |
| ATC CCC TTT GTC ATT GGG GGA AAC TTC ACT GGT AGC CAG AGG GCA GTC TTC CCG | | | |
| Ile Pro Phe Val Ile Gly Gly Asn Phe Thr Gly Ser Gln Arg Ala Val Phe Arg | | | |
| 500 | 515 | 530 | 545 |
| CAG GCC ATG AGG CAC TGG GAG AAG CAC ACC TGT GTC ACC TTC CTG GAG CGC ACT | | | |
| Gln Ala MET Arg His Trp Glu Lys His Thr Cys Val Thr Phe Leu Glu Arg Thr | | | |

560 575 590
 GAC GAG GAC AGC TAT ATT GTG TTC ACC TAT OGA CCT TGC GGG TGC TGC TCC TAC
 Asp Glu Asp Ser Tyr Ile Val Phe Thr Tyr Arg Pro Cys Gly Cys Cys Ser Tyr

605 620 635 650
 GTG GGT OGC OGC GGC GGG GGC CCC CAG GCC ATC TCC ATC GGC AAG AAC TGT GAC
 Val Gly Arg Arg Gly Gly Gly Pro Gln Ala Ile Ser Ile Gly Lys Asn Cys Asp

665 680 695
 AAG TTC GGC ATT GTG GTC CAC GAG CTG GGC CAC GTC GTC GGC TTC TGG CAC GAA
 Lys Phe Gly Ile Val Val His Glu Leu Gly His Val Val Gly Phe Trp His Glu

710 725 740 755
 CAC ACT OGC CCA GAC OGC GAC OGC CAC GTT TCC ATC GTT OGT GAG AAC ATC CAG
 His Thr Arg Pro Asp Arg Asp Arg His Val Ser Ile Val Arg Glu Asn Ile Gln

770 785 800 815
 CCA GGG CAG GAG TAT AAC TTC CTG AAG ATG GAG CCT CAG GAG GTG GAG TCC CTG
 Pro Gly Gln Glu Tyr Asn Phe Leu Lys MET Glu Pro Gln Glu Val Glu Ser Leu

830 845 860
 GGG GAG ACC TAT GAC TTC GAC AGC ATC ATG CAT TAC GCT OGC AAC ACA TTC TCC
 Gly Glu Thr Tyr Asp Phe Asp Ser Ile MET His Tyr Ala Arg Asn Thr Phe Ser

875 890 905 920
 AGG GGC ATC TTC CTG GAT ACC ATT GTC CCC AAG TAT GAG GTG AAC GGG GTG AAA
 Arg Gly Ile Phe Leu Asp Thr Ile Val Pro Lys Tyr Glu Val Asn Gly Val Lys

935 950 965
 CCT CCC ATT GGC CAA AGG ACA OGC CTC AGC AAG GGG GAC ATT GCC CAA GCC OGC
 Pro Pro Ile Gly Gln Arg Thr Arg Leu Ser Lys Gly Asp Ile Ala Gln Ala Arg

980 995 1010 1025
 AAG CTT TAC AAG TGC CCA GCC TGT GGA GAG ACC CTG CAA GAC AGC ACA GGC AAC
 Lys Leu Tyr Lys Cys Pro Ala Cys Gly Glu Thr Leu Gln Asp Ser Thr Gly Asn

1040 1055 1070 1085
 TTC TCC TCC CCT GAA TAC CCC AAT GGC TAC TCT GCT CAC ATG CAG TCC GTG TGG
 Phe Ser Ser Pro Glu Tyr Pro Asn Gly Tyr Ser Ala His MET His Cys Val Trp

1100 1115 1130
 OGC ATC TCT GTC ACA CCC GGG GAG AAG ATC ATC CTG AAC TTC ACG TCC CTG GAC
 Arg Ile Ser Val Thr Pro Gly Glu Lys Ile Ile Leu Asn Phe Thr Ser Leu Asp

1145 1160 1175 1190
 CTG TAC OGC AGC OGC CTG TGC TGG TAC GAC TAT GTG GAG GTC OGA GAT GGC TTC
 Leu Tyr Arg Ser Arg Leu Cys Trp Tyr Asp Tyr Val Glu Val Arg Asp Gly Phe

1205 1220 1235
 TGG AGG AAG GOG CCC CTC OGA GGC OGC TTC TGC GGG TCC AAA CTC CCT GAG CCT
 Trp Arg Lys Ala Pro Leu Arg Gly Arg Phe Cys Gly Ser Lys Leu Pro Glu Pro

1250 1265 1280 1295
 ATC GTC TCC ACT GAC AGC CGC CTC TGG GTT GAA TTC CGC AGC AGC AGC AAT TGG
 Ile Val Ser Thr Asp Ser Arg Leu Trp Val Glu Phe Arg Ser Ser Ser Asn Trp

 1310 1325 1340 1355
 GTT GGA AAG GGC TTC TTT GCA GTC TAC GAA GCC ATC TGC GGG GGT GAT GTG AAA
 Val Gly Lys Gly Phe Phe Ala Val Tyr Glu Ala Ile Cys Gly Gly Asp Val Lys

 1370 1385 1400
 AAG GAC TAT GGC CAC ATT CAA TCG CCC AAC TAC CCA GAC GAT TAC CGG CCC AGC
 Lys Asp Tyr Gly His Ile Gln Ser Pro Asn Tyr Pro Asp Asp Tyr Arg Pro Ser

 1415 1430 1445 1460
 AAA GTC TGC ATC TGG CGG ATC CAG GTG TCT GAG GGC TTC CAC GTG GGC CTC ACA
 Lys Val Cys Ile Trp Arg Ile Gln Val Ser Glu Gly Phe His Val Gly Leu Thr

 1475 1490 1505
 TTC CAG TCC TTT GAG ATT GAG CGC CAC GAC AGC TGT GCC TAC GAC TAT CTG GAG
 Phe Gln Ser Phe Glu Ile Glu Arg His Asp Ser Cys Ala Tyr Asp Tyr Leu Glu

1520 1535 1550 1565
 GTG CGC GAC GGG CAC AGT GAG AGC AGC ACC CTC ATC GGG CGC TAC TGT GGC TAT
 Val Arg Asp Gly His Ser Glu Ser Ser Thr Leu Ile Gly Arg Tyr Cys Gly Tyr

 1580 1595 1610 1625
 GAG AAG CCT GAT GAC ATC AAG AGC ACG TCC AGC CGC CTC TGG CTC AAG TTC GTC
 Glu Lys Pro Asp Asp Ile Lys Ser Thr Ser Ser Arg Leu Trp Leu Lys Phe Val

 1640 1655 1670
 TCT GAC GGG TCC ATT AAC AAA GCG GGC TTT GCC GTC AAC TTT TTC AAA GAG GTG
 Ser Asp Gly Ser Ile Asn Lys Ala Gly Phe Ala Val Asn Phe Phe Lys Glu Val

 1685 1700 1715 1730
 GAC GAG TGC TCT CGG CCC AAC CGC GGG GGC TGT GAG CAG CGG TGC CTC AAC ACC
 Asp Glu Cys Ser Arg Pro Asn Arg Gly Gly Cys Glu Gln Arg Cys Leu Asn Thr

 1745 1760 1775
 CTG GGC AGC TAC AAG TGC AGC TGT GAC CCC GGG TAC GAG CTG GCC CCA GAC AAG
 Leu Gly Ser Tyr Lys Cys Ser Cys Asp Pro Gly Tyr Glu Leu Ala Pro Asp Lys

1790 1805 1820 1835
 CGC CGC TGT GAG GCT GCT TGT GGC GGA TTC CTC ACC AAG CTC AAC GGC TCC ATC
 Arg Arg Cys Glu Ala Ala Cys Gly Gly Phe Leu Thr Lys Leu Asn Gly Ser Ile

 1850 1865 1880 1895
 ACC AGC CGC GGC TGG CCC AAG GAG TAC CCC CCC AAC AAG AAC TGC ATC TGG CAG
 Thr Ser Pro Gly Trp Pro Lys Glu Tyr Pro Pro Asn Lys Asn Cys Ile Trp Gln

 1910 1925 1940
 CTG GTG GCC CCC ACC CAG TAC CGC ATC TCC CTG CAG TTT GAC TTC TTT GAG ACA
 Leu Val Ala Pro Thr Gln Tyr Arg Ile Ser Leu Gln Phe Asp Phe Phe Glu Thr

1955 1970 1985 2000
 GAG GGC AAT GAT GTG TGC AAG TAC GAC TTC GTG GAG GTG OGC AGT GGA CTC ACA
 Glu Gly Asn Asp Val Cys Lys Tyr Asp Phe Val Glu Val Arg Ser Gly Leu Thr

2015 2030 2045
 GCT GAC TCC AAG CTG CAT GGC AAG TTC TGT GGT TCT GAG AAG CCC GAG GTC ATC
 Ala Asp Ser Lys Leu His Gly Lys Phe Cys Gly Ser Glu Lys Pro Glu Val Ile

2060 2075 2090 2105
 ACC TCC CAG TAC AAC AAC ATG OGC GTG GAG TTC AAG TCC GAC AAC ACC GTG TCC
 Thr Ser Gln Tyr Asn Asn MET Arg Val Glu Phe Lys Ser Asp Asn Thr Val Ser

2120 2135 2150 2165
 AAA AAG GGC TTC AAG GCC CAC TTC TTC TCA GAA AAG AGG CCA GCT CTG CAG CCC
 Lys Lys Gly Phe Lys Ala His Phe Phe Ser Glu Lys Arg Pro Ala Leu Gln Pro

2180 2195 2210
 CCT CGG GGA OGC CCC CAC CAG CTC AAA TTC CGA GTG CAG AAA AGA AAC OGC ACC
 Pro Arg Gly Arg Pro His Gln Leu Lys Phe Arg Val Gln Lys Arg Asn Arg Thr

2225 2235 2245 2255 2265 2275 2285
 CCC CAG TGAGGCCTGC CAGGCCTCCC GGACCCCTTG TTACTCAGGA ACCTCACCTT GGAOGGAATG
 Pro Gln

2295 2305 2315 2325 2335 2345 2355
 GGATGGGGGC TTOGGTGCCC ACCAACCCCC CACCTCCACT CTGCCATTCC GGCCACCTC CCTCTGGCOG

2365 2375 2385 2395 2405 2415 2425
 GACAGAACTG GTGCTCTCTT CTCCCCACTG TGCCOGTCCG OGGACOGGGG ACCCTTCCCC GTGCCCTACC

2435 2445 2455 2465 2475 2485 2495
 CCTTCCATT TTGATGGTGT CTGTGACATT TCCTGTGTGT AAGTAAAAGA GGGACCCCTG CGTCTGCCT

CTAGA

11. A process for producing BMP-2 Class I comprising culturing in a suitable culture medium a cell line transformed with a DNA sequence encoding BMP-2 Class I, said DNA sequence being in relative association with an expression control sequence therefor, and isolating BMP-2 Class I from said culture medium.

12. A process for according to Claim 11 wherein said DNA sequence comprises substantially the nucleotide sequence as

follows:

| | | | | | | | | | | | | | | | | | | |
|------------|------------|------------|------------|------------|------------|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 10 | 20 | 30 | 40 | 50 | 60 | 70 | | | | | | | | | | | | |
| GTGACTCTA | GAGTGTGTGT | CAGCACTTGG | CTGGGGACTT | CITGAACTTG | CAGGGAGAAT | AACTTGOGCA | | | | | | | | | | | | |
| 80 | 90 | 100 | 110 | 120 | 130 | 140 | | | | | | | | | | | | |
| CCCCACTTTG | OGGOGGTGCC | TTTGCCCCAG | OGGAGCCTGC | TTGGCCATCT | COGAGCCCCA | COGCCCCCTCC | | | | | | | | | | | | |
| 150 | 160 | 170 | 180 | 190 | 200 | 210 | | | | | | | | | | | | |
| ACTCCTGGGC | CTTGCCCGAC | ACTGAGAOGC | TGTTCCACGC | GTGAAAAGAG | AGACTGOGCG | GCOGGCACCC | | | | | | | | | | | | |
| 220 | 230 | 240 | 250 | 260 | 270 | 280 | | | | | | | | | | | | |
| GGGAGAAGGA | GGAGGCAAAG | AAAAGGAAOG | GACATTGGGT | CCITGOGCCA | GGTCCTTTGA | CCAGAGTTTT | | | | | | | | | | | | |
| 290 | 300 | 310 | 320 | 330 | 340 | 350 | | | | | | | | | | | | |
| TCCATGTGGA | OGCTCTTTCA | ATGGAOGTGT | CCCCOGGTGC | TTCTTAGAOG | GACTGOGGTC | TOCTAAAGGT | | | | | | | | | | | | |
| | 370 | | 385 | | 400 | | | | | | | | | | | | | |
| | OGACCATG | GTG | GCC | GGG | ACC | OGC | TGT | CIT | CTA | GCG | TTG | CTG | CTT | CCC | CAG | GTC | | |
| | MET | Val | Ala | Gly | Thr | Arg | Cys | Leu | Leu | Ala | Leu | Leu | Leu | Pro | Gln | Val | | |
| | 415 | | 430 | | 445 | | | | | | | | | | | | | |
| | CTC | CTG | GGC | GGC | GCG | GCT | GGC | CTC | GTT | COG | GAG | CTG | GGC | OGC | AGG | AAG | TTC | GCG |
| | Leu | Leu | Gly | Gly | Ala | Ala | Gly | Leu | Val | Pro | Glu | Leu | Gly | Arg | Arg | Lys | Phe | Ala |
| 460 | | 475 | | 490 | | 505 | | | | | | | | | | | | |
| GCG | GCG | TOG | TOG | GGC | OGC | CCC | TCA | TCC | CAG | CCC | TCT | GAC | GAG | GTC | CTG | AGC | GAG | |
| Ala | Ala | Ser | Ser | Gly | Arg | Pro | Ser | Ser | Gln | Pro | Ser | Asp | Glu | Val | Leu | Ser | Glu | |
| | 520 | | 535 | | 550 | | | | | | | | | | | | | |
| TTC | GAG | TTG | OGG | CTG | CTC | AGC | ATG | TTC | GGC | CTG | AAA | CAG | AGA | CCC | ACC | CCC | AGC | |
| Phe | Glu | Leu | Arg | Leu | Leu | Ser | MET | Phe | Gly | Leu | Lys | Gln | Arg | Pro | Thr | Pro | Ser | |
| | 580 | | 595 | | 610 | | | | | | | | | | | | | |
| AGG | GAC | GCC | GTG | GTG | CCC | CCC | TAC | ATG | CTA | GAC | CTG | TAT | OGC | AGG | CAC | TOG | GGT | |
| Arg | Asp | Ala | Val | Val | Pro | Pro | Tyr | MET | Leu | Asp | Leu | Tyr | Arg | Arg | His | Ser | Gly | |
| | 625 | | 640 | | 655 | | | | | | | | | | | | | |
| CAG | COG | GGC | TCA | CCC | GCC | CCA | GAC | CAC | OGG | TTG | GAG | AGG | GCA | GCC | AGC | OGA | GCC | |
| Gln | Pro | Gly | Ser | Pro | Ala | Pro | Asp | His | Arg | Leu | Glu | Arg | Ala | Ala | Ser | Arg | Ala | |
| | 685 | | 700 | | 715 | | | | | | | | | | | | | |
| AAC | ACT | GTG | OGC | AGC | TTC | CAC | CAT | GAA | GAA | TCT | TTG | GAA | GAA | CTA | CCA | GAA | ACG | |
| Asn | Thr | Val | Arg | Ser | Phe | His | His | Glu | Glu | Ser | Leu | Glu | Glu | Leu | Pro | Glu | Thr | |
| 730 | | 745 | | 760 | | 775 | | | | | | | | | | | | |
| AGT | GGG | AAA | ACA | ACC | CGG | AGA | TTC | TTC | TTT | AAT | TTA | AGT | TCT | ATC | CCC | ACG | GAG | |
| Ser | Gly | Lys | Thr | Thr | Arg | Arg | Phe | Phe | Phe | Asn | Leu | Ser | Ser | Ile | Pro | Thr | Glu | |
| | 790 | | 805 | | 820 | | | | | | | | | | | | | |
| GAG | TTT | ATC | ACC | TCA | GCA | GAG | CTT | CAG | GTT | TTC | OGA | GAA | CAG | ATG | CAA | GAT | GCT | |
| Glu | Phe | Ile | Thr | Ser | Ala | Glu | Leu | Gln | Val | Phe | Arg | Glu | Gln | MET | Gln | Asp | Ala | |

| | | | |
|---|------|------|------|
| | 850 | 865 | 880 |
| TTA GGA AAC AAT AGC AGT TTC CAT CAC CGA ATT AAT ATT TAT GAA ATC ATA AAA | | | |
| Leu Gly Asn Asn Ser Ser Phe His His Arg Ile Asn Ile Tyr Glu Ile Ile Lys | | | |
| 895 | 910 | 925 | 940 |
| CCT GCA ACA GCC AAC TCG AAA TTC CCC GTG ACC AGT CTT TTG GAC ACC AGG TTG | | | |
| Pro Ala Thr Ala Asn Ser Lys Phe Pro Val Thr Ser Leu Leu Asp Thr Arg Leu | | | |
| 955 | 970 | 985 | |
| GTG AAT CAG AAT GCA AGC AGG TGG GAA AGT TTT GAT GTC ACC CCC GCT GTG ATG | | | |
| Val Asn Gln Asn Ala Ser Arg Trp Glu Ser Phe Asp Val Thr Pro Ala Val MET | | | |
| 1000 | 1015 | 1030 | 1045 |
| CGG TGG ACT GCA CAG GGA CAC GCC AAC CAT GGA TTC GTG GTG GAA GTG GCC CAC | | | |
| Arg Trp Thr Ala Gln Gly His Ala Asn His Gly Phe Val Val Glu Val Ala His | | | |
| 1060 | 1075 | 1090 | 1105 |
| TTG GAG GAG AAA CAA GGT GTC TCC AAG AGA CAT GTT AGG ATA AGC AGG TCT TTG | | | |
| Leu Glu Glu Lys Gln Gly Val Ser Lys Arg His Val Arg Ile Ser Arg Ser Leu | | | |
| 1120 | 1135 | 1150 | |
| CAC CAA GAT GAA CAC AGC TGG TCA CAG ATA AGG CCA TTG CTA GTA ACT TTT GGC | | | |
| His Gln Asp Glu His Ser Trp Ser Gln Ile Arg Pro Leu Leu Val Thr Phe Gly | | | |
| 1165 | 1180 | 1195 | 1210 |
| CAT GAT GGA AAA GGG CAT CCT CTC CAC AAA AGA GAA AAA CGT CAA GCC AAA CAC | | | |
| His Asp Gly Lys Gly His Pro Leu His Lys Arg Glu Lys Arg Gln Ala Lys His | | | |
| 1225 | 1240 | 1255 | |
| AAA CAG CGG AAA CGC CTT AAG TCC AGC TGT AAG AGA CAC CCT TTG TAC GTG GAC | | | |
| Lys Gln Arg Lys Arg Leu Lys Ser Ser Cys Lys Arg His Pro Leu Tyr Val Asp | | | |
| 1270 | 1285 | 1300 | 1315 |
| TTC AGT GAC GTG GGG TGG AAT GAC TGG ATT GTG GCT CCC CCG GGG TAT CAC GCC | | | |
| Phe Ser Asp Val Gly Trp Asn Asp Trp Ile Val Ala Pro Pro Gly Tyr His Ala | | | |
| 1330 | 1345 | 1360 | 1375 |
| TTT TAC TGC CAC GGA GAA TGC CCT TTT CCT CTG GCT GAT CAT CTG AAC TCC ACT | | | |
| Phe Tyr Cys His Gly Glu Cys Pro Phe Pro Leu Ala Asp His Leu Asn Ser Thr | | | |
| 1390 | 1405 | 1420 | |
| AAT CAT GCC ATT GTT CAG ACG TTG GTC AAC TCT GTT AAC TCT AAG ATT CCT AAG | | | |
| Asn His Ala Ile Val Gln Thr Leu Val Asn Ser Val Asn Ser Lys Ile Pro Lys | | | |
| 1435 | 1450 | 1465 | 1480 |
| GCA TGC TGT GTC CCG ACA GAA CTC AGT GCT ATC TCG ATG CTG TAC CTT GAC GAG | | | |
| Ala Cys Cys Val Pro Thr Glu Leu Ser Ala Ile Ser MET Leu Tyr Leu Asp Glu | | | |

13. A process for producing BMP-2 Class II comprising culturing in a suitable culture medium a cell line transformed with a DNA sequence encoding BMP-2 Class II, said DNA sequence being in relative association with an expression control sequence therefor, and isolating BMP-2 Class II from said culture medium.

| | | | | | | |
|------------|-------------|------------|------------|------------|----------------|------------|
| 10 | 20 | 30 | 40 | 50 | 60 | 70 |
| CTCTAGAGGG | CAGAGGAGGA | GGGAGGGAGG | GAAGGAGOGC | GGAGCCOOGC | COGGAAGCTA | GGTGAGTGIG |
| 80 | 90 | 100 | 110 | 120 | 130 | 140 |
| GCATCOGAGC | TGAGGGAOGC | GAGCCTGAGA | OGCOGCTGCT | GCTCOGGCTG | AGTATCTAGC | TTGTCTCCCC |
| 150 | 160 | 170 | 180 | 190 | 200 | 210 |
| GATGGGATTC | COGTCCAAGC | TATCTOGAGC | CTGCAGOGCC | ACAGTCCCOG | GCCCTCGCCC | AGGTTCACTG |
| 220 | 230 | 240 | 250 | 260 | 270 | 280 |
| CAACOGTTCA | GAGGTCCCOCA | GGAGCTGCTG | CTGGOGAGCC | CGCTACTGCA | GGGACCTATG | GAGCCATTCC |
| 290 | 300 | 310 | 320 | 330 | 340 | 350 |
| GTAGTGCCAT | CCOGAGCAAC | GCACTGCTGC | AGCTTCCCTG | AGCCTTTCCA | GCAAGTTTGT | TCAAGATTGG |
| 360 | 370 | 380 | 390 | 400 | | |
| CTGTCAAGAA | TCATGGACTG | TTATTATATG | CCTTGTTTTC | TGTCAAGACA | CC ATG ATT CCT | |
| | | | | | MET Ile Pro | |

477 492 507
AGC CAT GCT AGT TTG ATA CCT GAG ACG GGG AAG AAA AAA GTC GCC GAG ATT CAG
Ser His Ala Ser Leu Ile Pro Glu Thr Gly Lys Lys Lys Val Ala Glu Ile Gln

[illegible]

1227 1242 1257 1272
 ACC TTT GGC CAT GAT GGC CCG GGC CAT GCC TTG ACC CGA CGC CCG AGG GCC AAG
 Thr Phe Gly His Asp Gly Arg Gly His Ala Leu Thr Arg Arg Arg Arg Ala Lys

 1287 1302 1317
 CGT AGC OCT AAG CAT CAC TCA CAG CCG GCC AGG AAG AAG AAT AAG AAC TGC CCG
 Arg Ser Pro Lys His His Ser Gln Arg Ala Arg Lys Lys Asn Lys Asn Cys Arg

 1332 1347 1362 1377
 OGC CAC TCG CTC TAT GTG GAC TTC AGC GAT GTG GGC TGG AAT GAC TGG ATT GTG
 Arg His Ser Leu Tyr Val Asp Phe Ser Asp Val Gly Trp Asn Asp Trp Ile Val

 1392 1407 1422 1437
 GCC CCA CCA GGC TAC CAG GCC TTC TAC TGC CAT GGG GAC TGC CCC TTT CCA CTG
 Ala Pro Pro Gly Tyr Gln Ala Phe Tyr Cys His Gly Asp Cys Pro Phe Pro Leu

 1452 1467 1482
 GCT GAC CAC CTC AAC TCA ACC AAC CAT GCC ATT GTG CAG ACC CTG GTC AAT TCT
 Ala Asp His Leu Asn Ser Thr Asn His Ala Ile Val Gln Thr Leu Val Asn Ser

 1497 1512 1527 1542
 GTC AAT TCC AGT ATC CCC AAA GCC TGT TGT GTG CCC ACT GAA CTG AGT GCC ATC
 Val Asn Ser Ser Ile Pro Lys Ala Cys Cys Val Pro Thr Glu Leu Ser Ala Ile

 1557 1572 1587
 TCC ATG CTG TAC CTG GAT GAG TAT GAT AAG GTG GTA CTG AAA AAT TAT CAG GAG
 Ser MET Leu Tyr Leu Asp Glu Tyr Asp Lys Val Val Leu Lys Asn Tyr Gln Glu

 1602 1617 1636 1646 1656 1666
 ATG GTA GTA GAG GGA TGT GGG TGC CCG TGAGATCAGG CAGTCCTTGA GCATAGACAG ATATACACAC
 MET Val Val Glu Gly Cys Gly Cys Arg

 1676 1686 1696 1706 1716 1726 1736
 CACACACACA CACCACATAC ACCACACACA CAGTTCCCA TCCACTCACC CACACACTAC ACAGACTGCT

 1746 1756 1766 1776 1786 1796 1806
 TCCTTATAGC TGCACITTTA TTTAAAAAAA AAAAAAATAA AATGGAAAAA ATCCCTAAAC ATTCACCTTG

 1816 1826 1836 1846 1856 1866 1876
 ACCITATTTA TGACTTTACG TGCAAATGTT TTGACCATAT TGATCATATA TTTTGACAAA ATATATTTAT

 1886 1896 1906 1916 1926 1936 1946
 AACTACGTAT TAAAACAAAA AAATAAAATG AGTCATTATT TTAAAAAATA AAAAAAACT CTAGAGTCCA

 CGGAATTC

15. A process for producing BMP-3 comprising culturing in a suitable culture medium a cell line transformed with a DNA sequence encoding BMP-3, said DNA sequence being in relative

association with an expression control sequence therefor and isolating BMP-3 from said culture medium.

16. A process according to Claim 15 wherein said DNA sequence comprises substantially the nucleotide sequence as follows:

| | | | | |
|--|------------|-------------|-------------------------------------|-----------------------------|
| 383 | 393 | 403 | 413 | 428 |
| GAGGAGGAAG | OGGTCTACGG | GGGTCTTCT | GOCTCTGCAG | AAC AAT GAG CTT CCT GGG GCA |
| | | | | Asn Asn Glu Leu Pro Gly Ala |
| 443 | 458 | 473 | 488 | |
| GAA TAT CAG TAC AAG GAG GAT GAA GTA TGG GAG GAG AGG AAG CCT TAC AAG ACT | | | | |
| Glu Tyr Gln Tyr Lys Glu Asp Glu Val Trp Glu Glu Arg Lys Pro Tyr Lys Thr | | | | |
| 503 | 518 | 533 | | |
| CTT CAG ACT CAG CCC CCT GAT AAG AGT AAG AAC AAA AAG AAA CAG AGG AAG GGA | | | | |
| Leu Gln Thr Gln Pro Pro Asp Lys Ser Lys Asn Lys Lys Lys Gln Arg Lys Gly | | | | |
| 548 | 563 | 578 | 593 | |
| CCT CAG CAG AAG AGT CAG ACG CTC CAG TTT GAT GAA CAG ACC CTG AAG AAG GCA | | | | |
| Pro Gln Gln Lys Ser Gln Thr Leu Gln Phe Asp Glu Gln Thr Leu Lys Lys Ala | | | | |
| 608 | 623 | 638 | | |
| AGA AGA AAG CAA TGG ATT GAA CCC OGG AAT TGT GCC AGA OGG TAC CTT AAA GTG | | | | |
| Arg Arg Lys Gln Trp Ile Glu Pro Arg Asn Cys Ala Arg Arg Tyr Leu Lys Val | | | | |
| 653 | 668 | 683 | 698 | |
| GAC TTC GCA GAT ATT GGC TGG AGC GAA TGG ATT ATT TCC CCC AAG TCC TTC GAT | | | | |
| Asp Phe Ala Asp Ile Gly Trp Ser Glu Trp Ile Ile Ser Pro Lys Ser Phe Asp | | | | |
| 713 | 728 | 743 | 756 | 766 |
| GCC TAT TAC TGC TCC GGA GCG TGC CAG TTC CCC ATG CCA AAG GTAGCCATTG TTTTGTGCC | | | | |
| Ala Tyr Tyr Cys Ser Gly Ala Cys Gln Phe Pro MET Pro Lys | | | | |
| 776 | 786 | | | |
| TGTCCTTCCC | ATTTCATAG | | | |
| ; and | | | | |
| 284 | 294 | 304 | 319 | |
| CTAACCTGTG | TTCTCCCTTT | TOGTTCCCTAG | TCT TTG AAG CCA TCA AAT CAC GCT ACC | |
| | | | Ser Leu Lys Pro Ser Asn His Ala Thr | |
| 334 | 349 | 364 | 379 | |
| ATC CAG AGT ATA GTG AGA GCT GTG GGG GTC GTC CCT GGA ATC CCC GAG CCT TGC | | | | |
| Ile Gln Ser Ile Val Arg Ala Val Gly Val Val Pro Gly Ile Pro Glu Pro Cys | | | | |
| 394 | 409 | 424 | 439 | |
| TGT GTG CCA GAA AAG ATG TCC TCA CTC AGC ATC TTA TTC TTT GAT GAA AAC AAG | | | | |
| Cys Val Pro Glu Lys MET Ser Ser Leu Ser Ile Leu Phe Phe Asp Glu Asn Lys | | | | |

454 469 484
 AAT GIG GTA CTT AAA GTA TAT CCA AAC ATG ACA GTA GAG TCT TGT GCT TGC AGA
 Asn Val Val Leu Lys Val Tyr Pro Asn MET Thr Val Glu Ser Cys Ala Cys Arg

 503 513 523 533
 TAACCTGGTG AAGAACTCAT CTGGATGCTT AACTCAATCG.

17. A cDNA sequence encoding BMP-1 comprising substantially the nucleotide sequence recited in Claim 10 or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-1.

18. A cDNA sequence encoding BMP-2 Class I comprising substantially the nucleotide sequence recited in Claim 12 or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-2 Class I.

19. A cDNA sequence encoding BMP-2 Class II comprising substantially the nucleotide sequence recited in Claim 14 or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-2 Class II.

20. A cDNA sequence encoding BMP-3 comprising substantially the nucleotide sequence recited in Claim 16 or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-3.

21. A vector containing a DNA sequence encoding an osteoinductive protein and heterologous DNA, the DNA sequence encoding the protein being selected from the group consisting of:

a. a DNA sequence encoding BMP-1 comprising substantially the nucleotide sequence recited in Claim 10 or a sequence which

hybridize thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-1;

b. a DNA sequence encoding BMP-2 Class I comprising substantially the nucleotide sequence recited in Claim 12 or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-2 Class I;

c. a DNA sequence encoding BMP-2 Class II comprising substantially the nucleotide sequence recited in Claim 14 or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-2 Class II; and

d. a DNA sequence encoding BMP-3 comprising substantially the nucleotide sequence recited in Claim 16 or a sequence which hybridizes thereto under stringent conditions and which upon expression codes for a protein exhibiting substantial properties of BMP-3.

22. A cell transformed with a vector according to claim 21 which is capable of expressing a DNA sequence encoding the osteoinductive protein and progeny of said cell.

23. The transformed cell according to claim 24 selected from the group consisting of a mammalian cell, a bacterial cell, an insect cell, and a yeast cell.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/01537

| | | |
|--|--|---|
| I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³ | | |
| According to International Patent Classification (IPC) or to both National Classification and IPC | | |
| IPC(4): C07K 13/00, 15/00; A61K 37/00; See Attachment | | |
| US CL: 530/350, 395, 397; 514/12; 536/27 See Attachment | | |
| II. FIELDS SEARCHED | | |
| Minimum Documentation Searched ⁴ | | |
| Classification System | Classification Symbols | |
| US | 530/350, 395, 397; 514/12; 536/27 435/68, 70, 172.3; 935/13 | |
| Documentation Searched other than Minimum Documentation to the extent that such Documents are Included in the Fields Searched ⁵ | | |
| COMPUTER SEARCH CAS, APS: BONE MORPHOGEN, BONE INDUCTIVE PROTEIN, BMP, OSTEOINDUCTIVE FACTOR | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴ | | |
| Category ⁶ | Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷ | Relevant to Claim No. ¹⁴ |
| X, P Y, P | US, A, 4,619,989 (URIST) 28 Oct 1986. | 1-8 9-20 |
| X Y | US, A, 4,563,350 (NATHAN ET AL) 7 January 1986. | 1-8 9-20 |
| X Y | US, A, 4,455,256 (URIST) 19 June 1984. | 1-8 9-20 |
| X — | Proc. Natl. Acad. Sci USA, Vol. 81, issued January 1984, (Washington, D.C.), | 1 — |
| Y | (URIST), "Purification of bovine morphogenetic protein by hydroxyapatite chromatography", pages 371-375. | 2-20 |
| <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁵ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div> | | |
| IV. CERTIFICATION | | |
| Date of the Actual Completion of the International Search ¹⁸ | | Date of Mailing of this International Search Report ¹⁹ |
| 08 October 1987 | | 20 OCT 1987 |
| International Searching Authority ¹ | | Signature of Authorized Officer ²⁰ |
| ISA/US | | Alvin E. Tanenholtz |

PCT/US87/01537

Attachment To Form PCT/ISA/210, Part I.

IPC(4): C12P 21/00, 21/02; C12N 15/00; C07H 15/12

US CL : 435/68, 70, 172.3; 935/13

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|--|---|------------------------------------|
| Category * | Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷ | Relevant to Claim No ¹⁸ |
| Y | <u>Science</u> , Vol. 220 issued 13 May 1983 (Washington, D.C.) (URIST) "Bone cell Differentiation and Growth Factors" pages 680-686. | 1-20 |
| Y | <u>Proc. Natl. Acad. Sci, USA</u> , Vol. 80 issued November 1983 (Washington, D.C.) (SAMPATH ET AL), "Homology of bone- inductive proteins from human monkey, bovine and rat extracellular matrix," pages 6591-6595. | 1-20 |
| Y | <u>Proc. Natl. Acad. Sci, USA</u> , Vol. 78 issued November 1981, (Washington, D.C.) (SUGGS ET AL), "Use of synthetic oligonucleotides as hybridization probes: Isolation of cloned cDNA sequence for human β_2 -microglobulin" pages 6613-6617. | 1-20 |